Abstract
Cancer stem cells are implicated in tumor progression, metastasis, and recurrence, although the exact mechanisms remain poorly understood. Here, we show that the expression of cellular prion protein (PrPc, PRNP) is positively correlated with an increased risk of metastasis in colorectal cancer. PrPc defines a subpopulation of CD44-positive cancer stem cells that contributes to metastatic capacity. PrPc+CD44+ colorectal cancer stem cells displayed higher metastatic capability, unlike PrPc−CD44− stem cells, that was inhibited by RNAi-mediated attenuation of PrPc. Notably, administration of PrPc monoclonal antibodies significantly inhibited tumorigenicity and metastasis of colorectal cancer stem cells in mouse models of orthotopic metastasis. PrPc promoted epithelial to mesenchymal transition (EMT) via the ERK2 (MAPK1) pathway, thereby conferring high metastatic capacity. Our findings reveal the function of PrPc in regulating EMT in cancer stem cells, and they identify PrPc as a candidate therapeutic target in metastatic colorectal cancer. Cancer Res; 73(8); 2682–94. ©2013 AACR.

Introduction
Cancer is a major cause of mortality, and most human cancer-related deaths are related to metastasis (1, 2). The difficulty in diagnosing and therapeutically eliminating metastasis is related to our limited understanding of the mechanisms specifically expressed on the surface of metastatic cancer cells. Accumulating evidence suggests that a subset of cancer cells, operationally termed cancer stem cells (CSC) or tumor-initiating cells, possesses enhanced ability to regenerate tumors and survive anticancer therapies (3). CSCs were first identified in the hematopoietic system (4), and have now been reported in most human solid tumors on the basis of their functional properties (5–7). Colorectal CSCs (CCSC) have been distinguished using CD44 or CD133 either alone (8–10), or combined with other markers, such as EpCAM, CD166, CD29, CD24, LGR5 and aldehyde dehydrogenase 1 (ALDH1; refs. 11–13). Previous studies by ourselves and other authors have shown that as few as 100 CD44+ cancer cells isolated from patients were able to develop into a heterogeneous tumor, and that spheroids derived from a single CD44+ cancer cell could recapitulate the heterogeneous hierarchy of tumor cells (8, 14). These studies indicate that CCSCs may trigger the formation of colorectal cancer (CRC), and that CD44 is a functionally important surface biomarker of CCSCs. Importantly, an antibody specific to CD44 selectively eradicated leukemic stem cells in vivo by altering the fate of leukemic stem cells (15). Monoclonal antibodies targeting surface antigens specifically expressed in CSCs offer a potentially powerful strategy to eliminate CSCs with minimal toxicity. Besides CD44, monoclonal antibodies targeting CD123 and CD47 have been reported to eradicate leukemia stem cells (16).

Recent evidence suggests that CSCs may be endowed with enhanced disseminating capacity enabling them to invade neighboring tissues or metastasize to distant organs (17–19). Although CD44 and some other adhesion molecules, such as E-cadherin, could act as migration suppressors by restricting cancer cells to their primary sites (20), these same molecules with certain modifications (e.g., splice variants of CD44) or in combination with other adhesion molecules, such as CD26, may result in metastatic phenotypes (21–24).
Cellular prion protein (PrPc) is a highly conserved glycoprotein present in all vertebrates and has the same protein sequence as the scrapie prion protein (PrPsc), a pathogenic factor in scrapie in sheep, bovine spongiform encephalopathy in cattle, and kuru in human beings (25, 26). In addition to the central involvement of PrPc in prion diseases, several lines of evidence have intrigued implicated PrPc in human tumors including glioblastoma, breast, prostate, gastric and colorectal cancers (CRC; 27). PrPc interacts homophilically with itself or heterotypically with components of the extracellular matrix (ECM) such as laminin and vitronectin, to mediate cell adhesion (28). PrPc expression is required for the self-renewal of CD34+ hematopoietic stem cells (29, 30). However, despite tantalizing evidence linking PrPc to cancer development remain elusive. Here, we report that high levels of PrPc expression (in PrPc+ cells) identify a functionally distinct subpopulation of CD44− CCSCs, which contributes to CRC initiation and metastasis. Our findings thus reveal hitherto-unknown roles of PrPc in CCSCs and epithelial–mesenchymal transition (EMT), and suggest that PrPc could provide a potential therapeutic target in metastatic CRCs.

Materials and Methods

Cells, animals, and samples

Fresh colon samples from patients with CRC and paired normal specimens were collected from the tumor bank of Beijing Cancer Hospital (Beijing, China), as approved by the Research Ethics Board at the Beijing Institute for Cancer Research. A tissue microarray (TMA) was constructed as described previously (31, 32). The NCI-60 human tumor cell line panel was kindly provided by Dr. Jacque Grassi (National Cancer Institute, Bethesda, MD). SW480 and MDA-MB-231 cell lines were purchased from American Type Culture Collection, authenticated using polyphasic (genotypic and phenotypic) testing every 6 months, and cultured according to the manufacturer’s instructions. Human primary CRC cells were isolated from patients and cultured in serum-free medium. All CCSCs were subcultured for fewer than 20 passages. Four-week-old female nude mice (BALB/c-nu-nu) and 5-week-old female Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Chinese Academy of Medical Sciences (Beijing, China), and all experiments were conducted under standard conditions in accordance with the National and Institutional regulations.

Tumorigenesis and metastasis in orthotopic xenograft models

NOD/SCID mice were anesthetized with pentobarbital and the cervices were exteriorized by laparotomy. Various numbers of CD44− PrPc−, CD44+ PrPc−, CD44+ PrPc+, and CD44− PrPc− subpopulations freshly sorted from patients, or 50,000 DsRed-labeled PrPc overexpressing or knockdown CCSCs were injected into the cecal wall. All the animals were monitored for up to 80 to 100 days or until tumor-associated death occurred. Primary tumors and metastasis were evaluated by a whole-body fluorescence imaging system (In-Vivo FX PRO, Carestream).

Results

The PrPc expression level is correlated with clinical and pathologic features and outcomes in CRC patients

On the basis of our previous observations that CD44 is a robust marker for CCSCs (14), we hypothesized that additional factors may exist that are coexpressed with CD44 in the promotion of migration and metastasis of CSCs. We therefore compared gene expression in colorectal cancer, liver metastases, and normal tissues by cDNA array conducted in our lab. In addition, we conducted a CD44 coexpression analysis from Oncomine datasets (Ki_Colon, Koinuma_Colon, and Smith_Colorectal_2 datasets; refs., 33–35). Interestingly, we found that several genes were in fact coexpressed with CD44 and upregulated in metastases; one of these genes was PRNP (data not shown), which encodes PrPc.

To find out whether PrPc and CD44 are associated with the clinical and pathologic features, we carried out immunohistochemical (IHC) staining in a TMA containing 309 colorectal tumor specimens. CD44 and PrPc were found to be coex pressed in most of the tumors, especially in those that were poorly differentiated (correlation coefficient = 0.73; Fig. 1A). The PrPc expression level was significantly higher in poorly differentiated than in moderately and well-differentiated samples (P < 0.001; Fig. 1A and Supplementary Fig. S1A), whereas the level of CD44 was not associated with clinical stage (P = 0.14; Fig. 1A). This observation was supported by examining multiple Oncomine cDNA microarray datasets, which revealed that the PRNP mRNA expression levels were significantly elevated in high-grade and advanced-stage CRCs (Supplementary Fig. S1B). Importantly, the patients with CRC with the highest PrPc expression were also found to have the poorest prognosis, with a median survival of only 12 months (Fig. 1B), which was significantly (P < 0.0001) shorter than that of patients whose primary tumor was PrPc-negative (median survival >5 years).

CRC-related death is usually attributable to recurrence and distant metastasis. Out of 309 specimens, there were 228 primary CRCs without metastasis versus 81 cases with concurrent distant metastasis. Strikingly, we found that PrPc was highly expressed in the primary tumors of the patients with liver or lung metastasis (P = 0.0007; Fig. 1C). Oncomine cDNA microarray data analysis also showed a close correlation of the PrPc level with disease recurrence and early patient death (Supplementary Fig. S1C). We also observed that PrPc expression was most prominent at the interface and invasion fronts of tumor nodules (identified by cytokeratin 20 staining; Fig. 1D), which may constitute a reservoir of mobile CSCs (36). In the lymph nodes that had been invaded by CRC cells, PrPc+ cells were also observed in vascular invasions (Fig. 1E and Supplementary Fig. S1D). In addition, high levels of PrPc were present in CRC cells and could be seen invading the tumor stroma (Fig. 1F). Collectively, these data indicate that high PrPc expression is associated with advanced clinical stages, increased metastatic risk, and reduced patient survival.
A subpopulation of PrPc⁺ CRC cells possesses CSC properties

We next examined PrPc and CD44 coexpression patterns at the cellular level, and determined the biologic and tumorigenic properties of PrPc-expressing cells in CRC. We detected CD44⁺ PrPc⁺ cells in the cryosections of CRCs by double immunofluorescence (IF) staining of CD44 and PrPc (Fig. 2A). Flow cytometry analysis revealed only a small percentage of PrPc⁺...
Figure 2. The CD44^+/PrPc^+ subpopulation of CRC cells possesses CSC properties. A, double immunofluorescence staining of CD44 (green) and PrPc (red) in a representative CRC cryosection. The section was counterstained with 4,6-diamidino-2-phenylindole (DAPI; blue). Scale bar, 100 μm. B, a representative 4-color flow cytometric analysis of a CRC. Cells were gated by CD45 negativity and EpCAM positivity to remove leukocytes and stromal cells (left and middle) before the indicated subpopulations were analyzed and sorted out on the basis of PrPc and CD44 expression (right). C, Venn diagram illustrating the degree of overlap between the CD44 and PrPc populations. The percentage of each subpopulation is shown with the median values (M) and ranges indicated. D, clonogenic efficiency of indicated subpopulations freshly isolated from CRC (day 42). Single colonies were magnified to show the detailed morphology. Colonies with diameter ≥0.5 mm were counted for statistical analysis. ***, P < 0.001; ****, P < 0.0001. E, representative H&E images of regenerated tumors from CD44^+/PrPc^+ and CD44^+/PrPc^- subpopulations in NOD/SCID mice (day 90). Note that the cancer cells from CD44^+/PrPc^- subpopulation have infiltrated into the underlying muscle tissue. Scale bar, 100 μm. F, growth curve of xenograft tumor initiated from 1 × 10^6 CD44 RNAi (black), PrPc RNAi (blue), CD44 and PrPc RNAi (bright red) CRC cells in nude mice (n = 6). Parental (brown) and scramble (pink) shRNA cells were used as controls. *, P < 0.01; ***, P < 0.001.
cells in the CD45+/EpCAM+/CD44+ population in patient tumors (Fig. 2B), and the abundance of CD44+/PrPc+ cells ranged from 0.02% to 10.83% (median 0.28%; Fig. 2C and Supplementary Table S1). Interestingly, the subpopulation of CD44+/PrPc- cells in the 6 patients with concomitant liver metastasis was significantly higher than that in the 25 primary CRC patients without metastasis (3.19% vs. 0.79%, mean, P = 0.03; Supplementary Table S1). Furthermore, PrPc and CD44 were closely associated in the NCI-60 human tumor cell line panel (correlation coefficient = 0.68; Supplementary Fig. S2A–S2C).

We next asked whether PrPc is associated with CD44 in contributing to CSC properties. The abilities to form anchorage-independent spheres/spheroids in vitro and to regenerate serially transplantable xenograft tumors in immunodeficient mice are 2 of the hallmarks of CSCs (37). We therefore used fluorescence-activated cell sorting (FACS) to purify 4 subpopulations: CD44+/PrPc+, CD44+/PrPc-, CD44-/PrPc+ and CD44-/PrPc- from patient tumors (Fig. 2B). The purity of each subpopulation was more than 97%, as assessed by post-sort FACS and fluorescence microscopic analyses (data not shown). As expected, CD44+/PrPc+ CRC cells displayed significantly greater ability to form colonies compared with CD44-/PrPc- or CD44-/PrPc+ cells (P = 0.02 and P = 0.007, respectively), whereas CD44-/PrPc- cancer cells failed to generate colonies (Fig. 2D).

To evaluate their tumorigenic potential, the CD44+/PrPc+, CD44+/PrPc-, CD44-/PrPc+ and CD44-/PrPc- subpopulations isolated from 9 patients (i.e., cases 22–30 and Supplementary Table S1) were subcutaneously injected into NOD/SCID mice. As predicted by their clonogenicity data, xenograft tumors were generated from the CD44+/PrPc+, CD44+/PrPc-, and CD44-/PrPc+ cells, but not from the CD44-/PrPc- cells in 6 of 9 cases (Supplementary Table S2). In limited-dilution assays, the CD44+/PrPc+ cells displayed greater tumor-initiating capacity than the other subpopulations (P < 0.001; Supplementary Table S2). We frequently observed that tumor cells had invaded the underlying muscle tissue in tumors regenerated from CD44+/PrPc+ (but not in those regenerated from CD44-/PrPc-) cells (Fig. 2E). To further test their self-renewal capabilities in vivo, 100 CD44+/PrPc+ cancer cells purified from a first-generation xenograft tumor were transplanted into NOD/SCID mice. Our findings indicated that 8 of 9 transplantsations grew secondary xenograft tumors that recapitulated the phenotype of the original tumor, and even higher tumorigenic capacities were detected in the third transplantation (Supplementary Fig. S2D and data not shown).

Similar tumor-initiating capacities were also observed in nude mice. We isolated the 4 subpopulations prepurified from CD45+ tumor cells, and injected them subcutaneously into nude mice (cases 1–11, Supplementary Table S1). Tumor development was observed in 8 cases (Supplementary Fig. S2E). Again, the CD44+/PrPc+ CRC cells exhibited the greatest tumor-initiating capacity (P < 0.05; Supplementary Table S2). As shown in Supplementary Figure S2E, the reconstituted tumors were histologically heterogeneous. PrPc and CD44 coexpression was detected in only a minor subset of cells in regenerated tumors (Supplementary Fig. S2F–S2G), also suggesting that the CD44+/PrPc+ cells were capable of self-renewal.

### Table 1. Tumor-initiating incidence and liver metastasis in the orthotopic model

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NOTE: Indicated numbers (500, 2,000 and 10,000) of CD44+/PrPc+, CD44+/PrPc-, CD44-/PrPc+, and CD44-/PrPc- subpopulations freshly isolated from CD45+/EpCAM+ CRC cells in 7 patients (i.e., case 24–30, Supplementary Table S1) were transplanted into cecal wall of NOD/SCID mice. In 6 of 7 cases, xenograft tumors were regenerated (day 100). The incidence of tumor regeneration at the cecum and the incidence of metastasis to the liver are shown.
Figure 3. PrPc confers greater metastatic capacity. A, 10,000 freshly sorted CD44\(^+\) PrPc\(^+\) cells from CRCs were transplanted into the cecal wall of NOD/SCID mice. The regenerated cecal tumor (black arrow) and the liver metastatic nodules (white arrow) are shown. B, metastatic nodules in the liver formed after injecting 2,000 of the cells into the cecal wall (day 100). Metastatic cancer cells are distinguished from normal mouse liver tissue by having a higher nucleus/cytoplasm ratio. Scale bar, 300 \(\mu\)m. C, metastatic efficiency. The number of cecal tumors with liver metastasis (white bar) vs. cecal tumors without metastasis (black bar) determined 100 days after CRC cell injections. D, metastatic areas were determined by Image-Pro Plus software based on H&E staining. The metastatic area was normalized to CD44\(^+\) PrPc\(^+\) cells. * * * \(P < 0.001\). E, PrPc\(^+\) cells exhibit a higher metastatic index than PrPc\(^-\) cells. Metastatic lesions were determined under a microscope on the basis of H&E staining, and the metastatic index was defined as the number of metastatic nodules per primary tumor volume. * * * \(P < 0.0001\). F, cell migration assays with the indicated subpopulations freshly isolated from CRCs. Each sample was analyzed in triplicate, and the experiment was carried out using 7 patient tumors. * * * \(P < 0.0001\). G, PrPc and CD44 expression in migrated vs. nonmigrated CRC cells determined by flow cytometry analysis. This experiment was conducted in triplicate using 6 patient tumors.
Figure 4. PrPc confers greater metastatic capacity. A, representative whole-body fluorescence imaging of orthotopically growing (dotted circles) and metastasizing CCSCs (n = 9, day 80). B, Western blot analysis of N-cadherin and E-cadherin in CCSCs in which PrPc was either overexpressed or knocked down. Monoglycosylated (30 kDa) and unglycosylated (28 kDa) PrPc expressed on CCSCs were detected by anti-PrPc (Clone6) antibody. C, double immunofluorescence staining of N-cadherin (green) and E-cadherin (red) in CCSCs in which PrPc was either overexpressed or knocked down. Scale bar, 50 μm. D, qRT-PCR analysis of EMT related genes in Twist or PrPc manipulated CCSCs. The mRNA expression levels were normalized to that of parental CCSCs. Purple bars, knock-in of twist in PrPc RNAi CCSCs. Yellow bars, knock-in of PrPc in Twist RNAi cells. **, P < 0.001; *** P < 0.0001. E, relative mRNA expression levels of N-cadherin, E-cadherin, and Twist in the indicated subpopulations freshly sorted from patients. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a common control. The mRNA expression levels of each gene were normalized to those of the cell population with the lowest mRNA expression. This experiment was conducted using cells from 6 patient tumors. ***, P < 0.001; ****, P < 0.0001. F, representative flow cytometry analysis showing that the N-cadherin-positive CRC cells were also PrPc positive, whereas most E-cadherin-positive cells were PrPc negative. This experiment was carried out in 8 freshly sorted CRCs by gating for CD44 EpCAM.

Figure 5. PrPc modulates EMT in CCSCs. A, phase-contrast images of CCSCs in which PrPc was either overexpressed or knocked down. Note the morphologic changes after the PrPc expression level was modulated. Scale bar, 50 μm. B, Western blot analysis of N-cadherin and E-cadherin in CCSCs in which PrPc was either overexpressed or knocked down. Monoglycosylated (30 kDa) and unglycosylated (28 kDa) PrPc expressed on CCSCs were detected by anti-PrPc (Clone6) antibody. C, double immunofluorescence staining of N-cadherin (green) and E-cadherin (red) in CCSCs in which PrPc was either overexpressed or knocked down. Scale bar, 50 μm. D, qRT-PCR analysis of EMT related genes in Twist or PrPc manipulated CCSCs. The mRNA expression levels were normalized to that of parental CCSCs. Purple bars, knock-in of twist in PrPc RNAi CCSCs. Yellow bars, knock-in of PrPc in Twist RNAi cells. **, P < 0.001; *** P < 0.0001. E, relative mRNA expression levels of N-cadherin, E-cadherin, and Twist in the indicated subpopulations freshly sorted from patients. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a common control. The mRNA expression levels of each gene were normalized to those of the cell population with the lowest mRNA expression. This experiment was conducted using cells from 6 patient tumors. ***, P < 0.001; ****, P < 0.0001. F, representative flow cytometry analysis showing that the N-cadherin-positive CRC cells were also PrPc positive, whereas most E-cadherin-positive cells were PrPc negative. This experiment was carried out in 8 freshly sorted CRCs by gating for CD44 EpCAM.
As in leukemic stem cells (15), knocking down CD44 in CRC cells significantly inhibited tumor regeneration (14). To investigate the role of PrPc in tumor initiation, we isolated primary CRC cells from patients and cultured them in ultra-low attachment dishes without serum. Remarkably, PrPc knockdown in the CRC cells also retarded tumor growth in nude mice, although the inhibitory effect was slightly less apparent than with CD44 knockdown (Fig. 2F). However, combined knockdown of both CD44 and PrPc completely abolished tumor development (Fig. 2F). Taken together, these data suggest that the CD44+/PrPc− subpopulation of CRC cells does possess CSC properties.

**PrPc+ CSC is more metastatic**

Because PrPc is highly expressed in metastatic tissues, we attempted to find out whether it contributes to metastasis in CRC. To recapitulate human cancers with greater fidelity, we transplanted 500, 2,000 and 10,000 cells from each of the CD44+/PrPc−, CD44+/PrPc+, CD44−PrPc−, and CD44−PrPc+ subpopulations isolated from 7 patients (i.e., case 24–30, Supplementary Table S1), into the cecal wall of NOD/SCID mice (38). As shown in Table 1, of the 6 patient-derived CRC cells produced cecal tumors, which was consistent with the results obtained from subcutaneously implanted tumor cells (Supplementary Table S2). The CD44+/PrPc− cells clearly formed tumors with the greatest efficiency ($P < 0.01$; Table 1 and Supplementary Table S2). Remarkably, macroscopic and microscopic analysis revealed that liver metastases were almost exclusively observed in the mice implanted with PrPc− cells (i.e., CD44+/PrPc− and CD44−PrPc−; Fig. 3A–C; Table 1). Furthermore, semiquantitative measurement of the metastatic area and the metastatic index confirmed that the tumors derived from PrPc− CRC cells were highly metastatic (Fig. 3D–E).

The spleen transplantation model is another commonly used approach to test the proliferative and metastatic potential of CSCs (39, 40). CD44+/PrPc−, CD44+/PrPc+, CD44−PrPc−, and CD44−PrPc+ subpopulations were sorted from the tumors of 10 patients (i.e., cases 12–21, Supplementary Table S1), and 50 cells from each subpopulation were then transplanted into the spleen of nude mice. Similar to the results obtained using the orthotopic model, liver metastasis occurred within 70 days in 28 of 30 cases of CD44+/PrPc−, 3 of 30 cases of CD44+/PrPc+, 15 of 30 cases of CD44−PrPc−, and 2 of 30 cases of CD44−PrPc+ cells (Supplementary Fig. S3A–S3B). These results suggest that PrPc− is intimately associated with the CRC liver metastasis. Transwell analysis showed that CD44+/PrPc− cells displayed about 16-fold greater migratory ability than CD44−PrPc− cells (Fig. 3F). Most of the migrating cells ($84.25 \pm 3.14\%$) were found to be PrPc-positive, versus only $1.25 \pm 0.22\%$ of the nonmigrant cells (Fig. 3G). Collectively, these observations imply that PrPc is crucial for CSCS migration in vitro and for metastasis in vivo.

**PrPc is functionally important for cell migration and cancer metastasis**

Because CSC is generally rare and it is difficult to label and trace its growth in animals, we generated a cell line of CD44+ CSCS isolated from a CRC patient. These cells have all the characteristics of CSCs including constitutive expression of CD44, and the capacity to form spheres and generate xenograft tumors (14). The CCSCs were grown in ultra-low attachment dishes without serum, and were subcultured once a week for fewer than 20 passages. To investigate the unique role of PrPc in metastasis, we used this cell line prelabeled with DsRed and in which PrPc had either been knocked down by shRNAs or was overexpressed via the pBABE-PURO retrovirus system. As shown in Fig. 4A–C, PrPc knockdown strongly inhibited tumor growth and liver metastasis as assessed by whole-body fluorescence imaging and hematoxylin and eosin (H&E) staining, suggesting that PrPc play a central role in CSC metastasis. In contrast, both tumor growth and liver metastasis were promoted by PrPc overexpression. Moreover, the level of PrPc expression was correlated with the metastatic index (Fig. 4D). Similar results were observed using the spleen metastasis model (Supplementary Fig. S4A–S4C). In addition, Transwell migration assays revealed that the levels of PrPc correlated with the migratory capacity of CCSCs (Fig. 4E). It is noteworthy that the PrPc levels did not alter the cell-cycle profiles (Supplementary Fig. S4D). Using CRC cells with CSC properties, we were able to show that PrPc is functionally required for the migration of CCSCs and contributes to CRC metastasis.

**PrPc antibodies inhibit migration and metastasis**

Surface markers unique to CSCs would provide ideal therapeutic targets on these cells, and indeed monoclonal antibodies have shown therapeutic efficacy in both preclinical tumor models and patients with cancer (41). We were therefore interested in addressing the question of whether PrPc could be used as a therapeutic target, in a manner similar to CD44 (15). We generated several monoclonal antibodies against PrPc, out of which we selected the IgG1 antibody. The specificity of the antibodies was verified by ELISA, Western blotting (WB), and IHC staining (Supplementary Fig. S4E–S4G). The migration of CD44+ CCSCs in vitro was strongly inhibited after they had been treated with 1 μg/ml anti-PrPc antibodies. The inhibitory effect obtained was greater than that associated with the anti-CD44 antibody (~1.7 fold). A combination of 1 μg/ml anti-CD44 and 1 μg/ml anti-PrPc antibodies was able to inhibit migration by up to approximately 80% (Fig. 4F); whereas PrPc
antibodies did not show any effect on cell migration in PrPc knockdown cells (data not shown).

It was essential to find out whether PrPc monoclonal antibodies can prevent CRC metastases in vivo. NOD/SCID mice bearing orthotopic implantations of CCSCs were administered 200 μg antibodies intraperitoneally on a weekly basis. ELISA analysis revealed that the concentration of anti-PrPc antibody in the serum of tumor-bearing animals was maintained at 0.87 to 1 μg/ml (data not shown). The orthotopic and metastatic tumors were monitored by whole-body fluorescence imaging every week and after 8 to 10 weeks the mice were sacrificed and underwent pathology examinations. The presence of anti-PrPc antibodies dramatically decreased tumorigenicity and liver metastases (Fig. 4G), whereas their body weight did not decrease (not shown). CCSCs precoated with anti-PrPc antibodies before orthotopic implantation produced a similar inhibitory effect (Supplementary Fig. S4H).

To make sure that the observed metastasis-inhibiting effects of anti-PrPc antibodies were not due to inhibition of primary tumor growth, we preinoculated the CCSCs orthotopically into the cecal wall for 3 weeks. Mice with equivalent tumors were then grouped and injected with 100 or 200 μg antibodies weekly. The results clearly showed that the anti-PrPc antibody inhibited tumor growth and metastasis at the lower dosage (100 μg/week) and eliminated tumor growth at higher dosage (200 μg/week; Supplementary Fig. S4H).

**PrPc promotes EMT in CCSCs**

We consistently observed that the PrPc-overexpressing CCSCs exhibited an elongated mesenchymal-like morphology, whereas the PrPc knockdown cells were flatter (Fig. 5A; Supplementary Fig. S4G). Also, PrPc⁺ CRC cells generated branching colonies with loose cell–cell interaction (see Fig. 2D), characteristic of the EMT phenotype. This prompted us to investigate whether PrPc promotes EMT, a known mechanism of CSC migration and invasion. Cells undergoing EMT retain stem cell functionality and form invasive front colonies (42). We first used quantitative reverse transcription-PCR (qRT-PCR; primers shown in Supplementary Table S3) to measure several EMT-related molecules (Fig. 5B and C; Supplementary Fig. S5B), which may have contributed to the metastasis observed in PrPc overexpressing cells. In contrast, knockdown of PrPc resulted in a reversed expression pattern (Supplementary Fig. S5A). Western blot analysis and double immunofluorescence staining further confirmed the correlation of PrPc with the expression of EMT-related molecules (Fig. 5B and C; Supplementary Fig. S5B).

Twist is a key transcription factor regulating mesenchymal cell fate, differentiation, and morphogenesis (43). Because PrPc expression had been found to influence the expression of Twist (Supplementary Fig. S5A), we reasoned that PrPc might regulate EMT via Twist. To test this, Twist was ectopically expressed in CCSCs in which PrPc was stably knocked down by shRNA. We observed that constitutively overexpressed Twist was indeed able to induce EMT in PrPc-knocked down CCSCs, leading to increased levels of Snail and N-cadherin (Fig. 5D). However, overexpression of PrPc in the Twist RNAi cells was unable to restore EMT, probably because Snail and N-cadherin expression was lower than in the parental cells (Fig. 5D). Overall, we conclude that PrPc regulates the EMT phenotype by modulating Twist.

Next, we used qRT-PCR to measure the mRNA levels of Twist, N-cadherin, and E-cadherin in CD44⁺ PrPc⁺, CD44⁺ PrPc⁻, CD44⁺ PrPc⁺, and CD44⁺ PrPc⁻ subpopulations of CRC cells freshly purified from 6 patients. The results showed that both Twist and N-cadherin were expressed at significantly higher levels in the PrPc-positive (i.e. CD44⁺ PrPc⁺ and CD44⁺ PrPc⁻) CRC cells than in the PrPc⁻ subpopulations (Fig. 5E). Furthermore, approximately 82% of N-cadherin⁺ cells were also PrPc⁺, whereas more than 93% of E-cadherin⁺ cells were PrPc⁻ in CD45⁺ EpCAM⁺ CRC cells as analyzed by flow cytometry (Fig. 5F). These findings suggest that PrPc is positively correlated with the mesenchymal properties of cells from CRC patients.

**Evidence that PrPc-induced EMT is ERK2 dependent**

**ERK** is regulated by the TGF-β and ERK signaling pathways (44). To understand the molecular mechanisms of PrPc-induced EMT, we first assessed the activation of these 2 pathways (45). Knocking down PrPc seemed to result in a reduction of both Phospho-smad2 and ERK1/2; however, overexpression of PrPc activated ERK1/2 but not smad2, suggesting that ERK signaling may act downstream of PrPc (Fig. 6A). In support of this, we found that U0126, an inhibitor of ERK signaling, suppressed the expression of several EMT-associated molecules (Fig. 6B) as well as EMT itself, especially when PrPc was overexpressed.

Laminin is a PrPc ligand (46), which, we reasoned, could activate events occurring downstream of PrPc, such as EMT. Laminin did enhance both ERK2 phosphorylation and the EMT phenotype (Fig. 6C and D). Interestingly, PrPc antibody blocked the laminin-induced ERK activation, EMT protein expression and EMT morphology in a dose-dependent manner (Fig. 6C and D), probably via interference with the interaction between laminin and its receptor PrPc. Immunofluorescence staining confirmed that the anti-PrPc antibody attenuated the laminin-induced N-cadherin expression (Fig. 6E). In addition, Transwell migration assays revealed that U0126 blocked the PrPc overexpression-induced migration, and that the anti-PrPc antibody also attenuated laminin-enhanced migration (Fig. 6F). Our findings thus suggest that ERK2 signaling pathway is involved in PrPc-induced EMT.

**Discussion**

It has been shown that CSCs contribute to both tumor initiation and cancer metastasis in many tumor systems. However, in most cases, the underlying mechanisms are not well understood. In this study, we have identified a distinct PrPc⁺ subpopulation in CD44⁺ CCSCs that displays enhanced capacity to regenerate tumors. These results emphasize the functionalities of PrPc as an "enrichment" factor for CCSC. Our data show that PrPc is a novel CCSC biomarker, which, by itself,
functionally contributes to both CRC tumorigenicity and metastasis. Interestingly, the PrPc− CRC cells tend to localize at the invasion fronts in patient tumors, are more mesenchymal, and behave like other cancer cells undergoing EMT, properties that have probably given these cells a greater capacity to disseminate and metastasize. These observations indicate PrPc's role in regulating CCSC likely occurring through its ability to promote EMT and the exact mechanism warrants future investigations. In terms of the mechanism involved, our work implies that PrPc may regulate EMT through ERK2- and Twist-dependent signaling pathways. Importantly, the interaction of PrPc with its ligand, laminin, directly activates ERK2 signaling and promotes EMT. Therefore, conceptually, interference with PrPc expression/functional molecules such as PrPc and CD26, which in turn mediate the interactions of CSCs with their microenvironment. Future work will attempt to elucidate the potential relationship between PrPc− CRC and CD26− CCSCs.

Previous studies have shown that the expression of PrPc is positively correlated with invasiveness and drug resistance in both gastric and breast cancers (27, 49). In addition, Liao and colleagues have reported that endoglin and PrPc are enriched in both gastric and breast cancers (27, 49). In addition, Liao and colleagues have reported that endoglin and PrPc are enriched in both gastric and breast cancers (27, 49).

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

Authors' Contributions
Conception and design: L. Du, B. Laffin, M. Mehrpour, Q. Chen
Development of methodology: L. Du, G. Rao, B. Li, Y. Zhu, D.G. Tang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Rao, H. Wang, B. Li, W. Tian, J.T. Cui, L. He, X. Tian, C. Hao, X. Liu, Y. Zhu, D.G. Tang, X. Lu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Du, G. Rao, H. Wang, B. Li, B. Laffin, D.G. Tang, M. Mehrpour
Writing, review, and/or revision of the manuscript: L. Du, G. Rao, B. Li, M. Mehrpour, Y. Lu, Q. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Rao, B. Li, X. Sun, D.G. Tang
Study supervision: Y. Lu, Q. Chen

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References


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