SHORT COMMUNICATION

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Paired related homoeobox 1, a new EMT inducer, is involved in metastasis and poor prognosis in colorectal cancer

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Background: Paired related homoeobox 1 (*PRRX1*) has been identified as a new epithelial-mesenchymal transition (EMT) inducer in breast cancer. However, the function of *PRRX1* in colorectal cancer (CRC) has not been elucidated.

Methods: We utilised ectopic *PRRX1*-expressing cell lines to analyse the function of *PRRX1* in CRC. The clinical significance of PRRX1 was also examined on three independent CRC case sets.

Results: *PRRX1* induced EMT and the stem-like phenotype in CRC cells. In contrast to studies of breast cancer, abundant expression of PRRX1 was significantly associated with metastasis and poor prognosis in CRC.

Conclusion: *PRRX1* is an indicator of metastasis and poor prognosis in CRC cases. Further investigation is required to uncover the signalling network regulating *PRRX1*.

The first event in cancer metastasis is the movement of cancer cells away from the primary tumour tissue. Thereafter, cancer cells invade surrounding tissues and intravasate into vessels. Several reports have shown that the invasive capability of cancer cells is acquired by transformation to the mesenchymal phenotype (the epithelial-mesenchymal transition (EMT; Nieto, 2011). Moreover, EMT may produce cancer stemness properties (Alison *et al*, 2011). Inducers such as TWIST1, (Kang and Massague, 2004) SNAI1, (Pena *et al*, 2009) ZEB1, and ZEB2 (Chua *et al*, 2007) are reported to be associated with EMT in cancer cells.

Most recently, Ocana *et al* (2012) reported that a newly identified EMT inducer, paired related homoeobox 1 (*PRRX1*), promoted full EMT in cancer cells. However, they focused on the mesenchymal-to-epithelial transition (MET; Thiery *et al*, 2009) They showed that the loss of *PRRX1* was required for cancer metastasis. Downregulation of *PRRX1* caused the acquisition of MET. Low *PRRX1* expression levels were significantly associated with metastasis and poor prognosis in the analysis of clinical samples of breast cancer and lung squamous cell carcinoma. Furthermore, the stemness phenotype was suppressed by *PRRX1*-induced EMT, and *PRRX1* had to be downregulated to activate stem cell properties and allow colonisation. These findings were clearly distinct from previous analyses of EMT and the stemness phenotype. We noted that the function of *PRRX1* in colorectal cancer (CRC) cells had not been elucidated. Thus, the purpose of the present study was to verify that *PRRX1* induced EMT in CRC cells. We also examined the clinical significance of *PRRX1* in CRC cases.

MATERIALS AND METHODS

Patients and sample collection. A total of 173 CRC samples were obtained during surgery. All patients underwent resection of the primary tumour at Kyushu University Beppu Hospital and affiliated hospitals between 1992 and 2007. Full informed consent was obtained from all individuals according to the local ethical

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review board approval. Detailed information is described in the Supplementary Material.

Cell lines. The human CRC cell lines DLD-1 and COLO-205 were obtained from the Japanese Cancer Research Bank (Tokyo, Japan) and maintained in RPMI 1640 medium containing 10% foetal bovine serum, 100 units per ml penicillin, and 100 μg ml^{-1} streptomycin sulphate. All cells were cultured in a humidified 5% CO_2 incubator at 37 °C.

***PRRX1* expression lentiviral vector.** To generate *PRRX1* expression lentiviral vectors, we amplified the insert (full-length human *PRRX1;* NM_022716.2) by PCR from human reference cDNA. Lentiviruses were produced by transient transfection of HEK293T

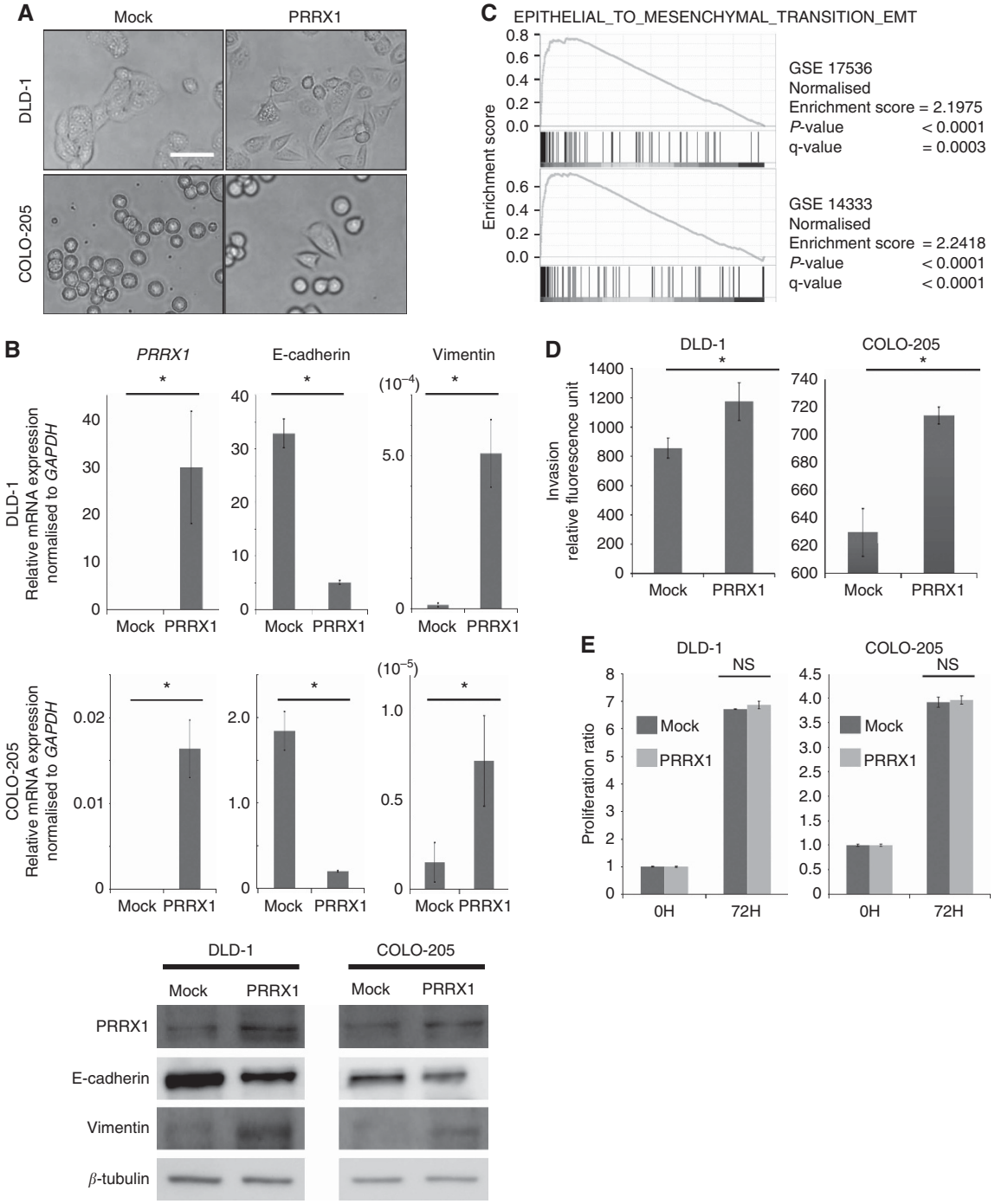

Figure 1. *PRRX1* expression supports an invasive and mesenchymal phenotype in CRC cells. (**A**) Phase-contrast images showing the phenotype of mock-transfected cells of DLD-1 and COLO-205 cells or of those in which *PRRX1* has been ectopically expressed. Scale bars: 100 μm. (**B**) Real-time RT–PCR (upper panel, DLD-1; middle panel, COLO-205) and western blot (lower panel: left, DLD-1; right, COLO-205) analyses of mock and *PRRX1*-expressing cells show the repression of E-cadherin and the activation of vimentin following *PRRX1* ectopic expression. (**C**) Enrichment plots of gene expression signatures for 'EPITHELIAL_TO_MESENCHYMAL_TRANSITION_EMT' according to *PRRX1* expression levels with data sets, GSE17536, and GSE14333. (**D**) Invasive properties of DLD-1 cells (left panel) and COLO-205 cells (right panel), with or without *PRRX1* expression. (**E**) Cell proliferation ratio of DLD-1 cells (left panel) and COLO-205 cells (right panel) with or without PRRX1 expression. Histograms represent the means ± s.d. of three independent experiments. *statistically significant ($P \leqslant 0.05$).

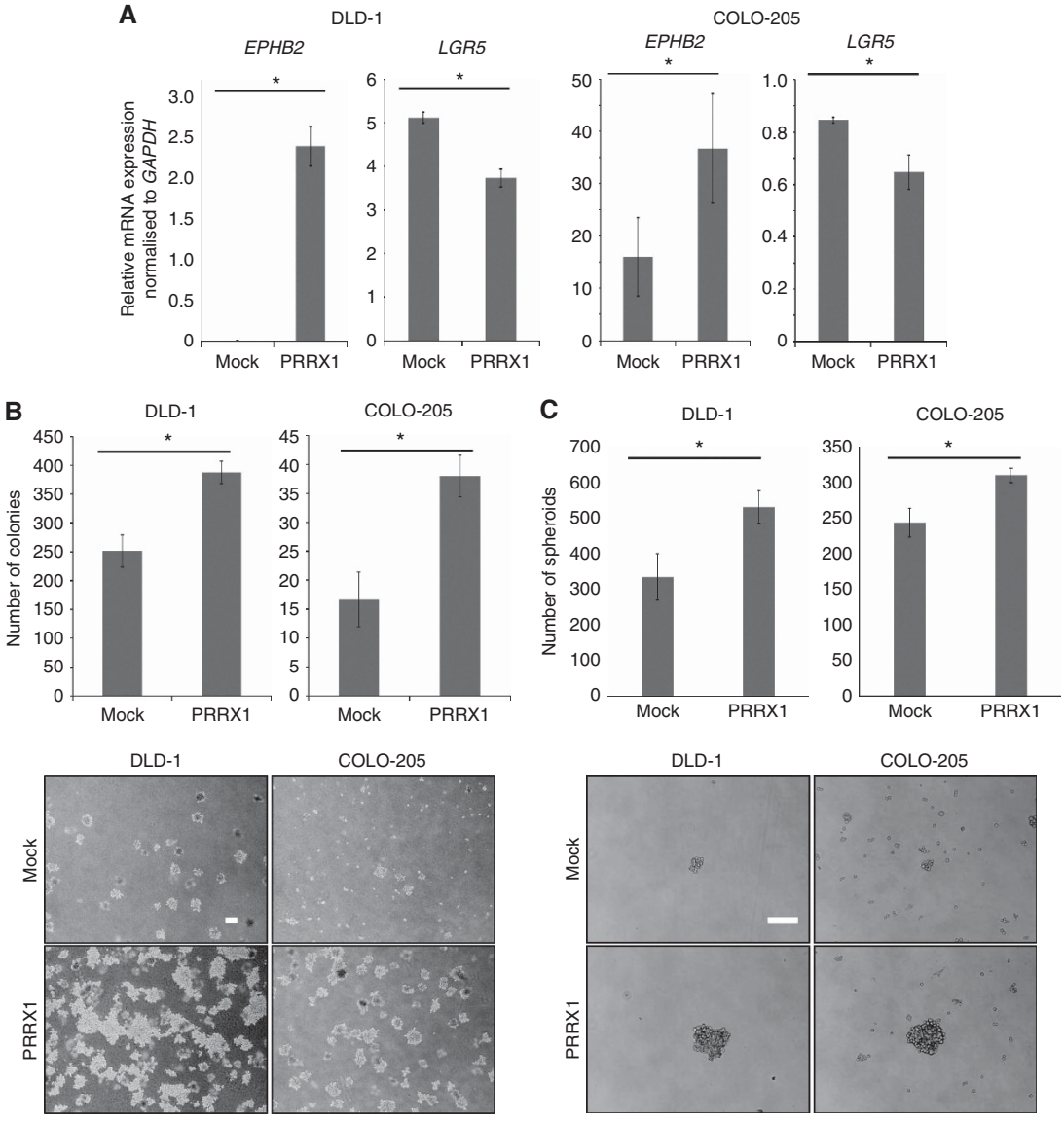

Figure 2. *PRRX1* led CRC cells to express stem-like properties. (**A**) Real-time RT–PCR (upper panel, DLD-1; lower panel, COLO-205) analyses of mock and PRRX1-expressing cells on *EPHB2* and *LGR5*, representative CRC stem cell markers. (**B**) Upper panel, quantitation of the soft agar colony-formation assay. Lower panel, representative images depicting colonies formed by mock and PRRX1-expressing cells. Scale bars: 100 μm. (**C**) Upper panel, sphere formation assay. Lower panel, representative images depicting spheroids formed by mock and PRRX1-expressing cells. Scale bars: 100 μm. Histograms represent the means ± s.d. of three independent experiments. *statistically significant ($P \leqslant 0.05$).

cells with pCMV-VSV-G-RSV-Rev, pCAG-HIVgp, and either CSII-CMV-HOTAIR or CSII-CMV-MCS (empty) plasmid DNAs (5′-*Xho*I and 3′-*Eco*RI sites) plus Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Quantitative real-time reverse-transcription (qRT)-PCR. qRT-PCR was performed in a LightCycler 480 instrument (Roche Applied Science, Basel, Switzerland) using a LightCycler 480 Probes Master kit or LightCycler 480 SYBR Green I Master kit (Roche Applied Science), following the manufacturer's protocol. The detailed protocol and the primer sequences used here are described in the Supplementary Material and Supplementary Table 1.

Immunoblotting. Total protein was extracted from *PRRX1*-expressing cell lines and mock cell lines. Aliquots of total protein (40 μg) were electrophoresed in 10% concentrated polyacrylamide gel, and then electrophoresed and electroblotted as previously described (Ieta *et al*, 2007). Detailed information can be found in the Supplementary Material.

Cell proliferation and cancer cell invasion assays. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche Applied Science) was used to evaluate cell proliferation. The BD BioCoat Tumor Invasion System, 8 μm pores (BD Bioscience, San Jose, CA, USA), was used to evaluate invasive capacity, following the manufacturer's protocol. Detailed information is provided in the Supplementary Material.

Soft agar and sphere formation assay. Detailed information is provided in the Supplementary Material.

Meta-analysis and gene set enrichment analysis. We obtained CRC expression profiles from the National Center for Biotechnology Information Gene Expression Omnibus database (accession codes GSE17536 and GSE14333) and analysed these expression profiles using gene set enrichment analysis

(GSEA; Subramanian *et al*, 2005). Detailed information is available in the Supplementary Material.

Statistical analysis. For continuous variables, data were expressed as means ± s.d. The relationship between *PRRX1* mRNA expression and clinicopathological factors was analysed using a χ^2-test and a Student's *t*-test. Findings were considered significant when the *P*-value was <0.05. All tests were performed using JMP software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Ectopic *PRRX1* promoted the EMT in CRC cells. To examine whether *PRRX1* induced EMT in CRC cells, we utilised ectopic *PRRX1*-expressing CRC cell lines. *PRRX1*-expressing cells showed a more spindle-like shape than did mock cells (Figure 1A). Both *PRRX1*-expressing cell lines had a significantly lower level of E-cadherin gene expression and a higher level of vimentin gene expression than did mock cells (Figure 1B). We also analysed the expression of well-known EMT transcription factors (Supplementary Figure 1). The data revealed that ectopic expression of *PRRX1* significantly affected the expression of EMT transcription factors *TWIST1* and *ZEB1*, but the directionality of the changes was not consistent between the cell lines. However, GSEA on two published clinical data sets of CRC (GSE17536 (Smith *et al*, 2010) and GSE14333 (Jorissen *et al*, 2009)) suggested that *PRRX1* expression was significantly correlated with the EMT signature (Figure 1C). To further investigate the participation of *PRRX1* in cancer invasiveness, invasion assays were carried out. *PRRX1*-expressing cells were more invasive than were mock cells in both cell lines (Figure 1D). On the other hand, cell proliferation was not affected by ectopic PRRX1 expression (Figure 1E). Our data indicated that *PRRX1* enhanced the invasive CRC phenotype via EMT induction *in vitro*.

Ectopic *PRRX1* promoted the stemness phenotype and anchorage-independent growth of CRC cells. With regard to the previously reported stemness properties induced by loss of *PRRX1* (Ocana *et al*, 2012), we verified the association between expression of *PRRX1* and representative CRC stem cell markers (Barker *et al*, 2009; Merlos-Suarez *et al*, 2011). Ectopic *PRRX1* expression upregulated *EPHB2*, an intestinal stem cell marker, but *LGR5* was adversely decreased (Figure 2A). Next, a sphere formation assay was carried out to investigate the impact of *PRRX1* on the stem-like phenotype of CRC cells. *PRRX1*-expressing cell lines showed a significant increase in colony formation compared with mock cells (Figure 2B). Further, soft agar colony-formation assays revealed the higher capability of anchorage-independent growth of *PRRX1*-expressing cells compared with mock cells (Figure 2C). Therefore, the current findings indicated that *PRRX1* induced the stemness phenotype in CRC in at least one pathway.

Abundant *PRRX1* expression was correlated with poor prognosis and metastasis in CRC cases. To determine how *PRRX1* expression influenced cancer metastasis and disease prognosis in CRC, we analysed the relationship between the *PRRX1* expression level, clinicopathological factors, and prognostic data. *PPRX1* mRNA expression levels of 173 CRC tissues were assessed by qRT-PCR. Cases were subdivided into two groups according to *PRRX1* mRNA expression level by the minimum *P*-value approach, which is a comprehensive method to find the optimal risk separation cut-off point in continuous measurements (Mizuno *et al*, 2009). The *PRRX1* high expression group ($N=64$) had a significantly poorer overall survival rate than did the *PRRX1* low expression group ($N=109$) (Figure 3A). Clinicopathological factors were compared between the high and low *PRRX1* mRNA expression groups (Supplementary Table 2). The high *PRRX1* expression group showed a higher risk for invasion. In addition, univariate and multivariate analyses of lymph node metastasis revealed that

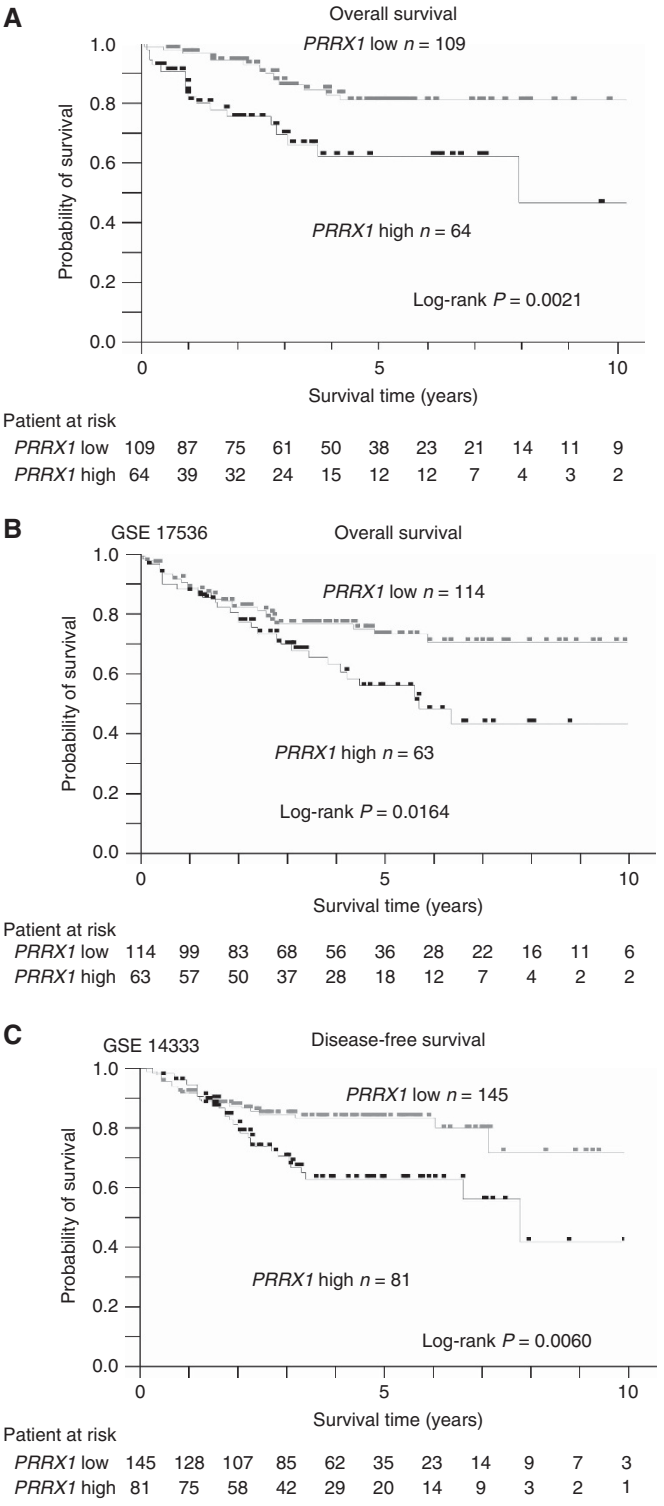

Figure 3. Abundant *PRRX1* expression results in higher metastasis rate and poor prognosis in CRC cases. (**A**) Kaplan–Meier overall survival curves for 173 patients with CRC according to *PRRX1* mRNA expression. (**B**) Kaplan–Meier overall survival curves for 177 patients with CRC (GSE17536) according to *PRRX1* expression. (**C**) Kaplan–Meier disease-free survival curves for 226 patients with CRC (GSE 14333) according to *PRRX1* expression. *statistically significant ($P\leqslant0.05$).

the *PRRX1* expression level was an independent indicator for lymph node metastasis (Supplementary Table 3). However, there was a discrepancy between our data on CRC and those of Ocana *et al* (2012) who showed that the low expression group had a significantly poorer prognosis in breast cancer and lung squamous cell carcinoma. We also analysed two other independent data sets described above (GSE17536 and GSE14333). In accordance with our results, the high *PRRX1* expression group showed poorer prognosis than did the low *PRRX1* expression group in both data sets (Figures 3B and 2C).

DISCUSSION

We have shown that *PRRX1*, a newly identified EMT inducer, enhances the mesenchymal and stem-like phenotype of CRC cells. Moreover, high expression of *PRRX1* was significantly correlated to metastasis and poor prognosis. Our results on the relationship between *PRRX1* expression and cancer prognosis differ substantially from those of Ocana *et al* (2012). Thus, our results suggest that *PRRX1* expression is associated with different phenotypes in different types of cancer cells. For example, breast cancer cells acquire MET and stem-like phenotypes by loss of *PRRX1*(Ocana *et al*, 2012). In contrast, CRC cells show EMT and stem-like phenotypes when they express *PRRX1*. Our data on CRC agree with a very recent report that showed that *PRRX1* has a central role in pancreatic regeneration and carcinogenesis (Reichert *et al*, 2013). In the current study, two *PRRX1*-expressing cell lines differed in their expression profiles of EMT transcription factors (Supplementary Figure 1). We speculate that this is because *PRRX1* induces EMT independently of classical EMT pathways as shown by Ocana *et al* (2012). The pathways activating EMT and stemness properties in intestinal tissue (Yan *et al*, 2012; Zhu *et al*, 2013) might be diverse, and further studies are required.

In the current study, we focused on expression of *PRRX1,* a gene that regulates EMT and stem cell properties. Our results demonstrated different roles for *PRRX1* in CRC from those in breast cancer. It has been suggested that transforming growth factor-β (Ocana *et al*, 2012) and microRNA (Yang *et al*, 2013) regulate the expression of *PRRX1*. However, details are lacking and further analysis is required to uncover the signalling network regulating *PRRX1*. Such data will be important for the development of new therapeutic approaches to control cancer metastases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)