FULL PAPER

BJC

British Journal of Cancer (2016) 115, 1367–1378 | doi: 10.1038/bjc.2016.362

Keywords: *miR-214*; *FOXD3*; *MED19*; metastasis; methylation; colorectal cancer

The *FOXD3*/miR-214/*MED19* axis suppresses tumour growth and metastasis in human colorectal cancer

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Background: *MiR-214* is aberrantly regulated in several tumours, but its underlying mechanisms in colorectal cancer (CRC) metastasis remain largely unknown. This study aimed to demonstrate the function and potential mechanism of *miR-214* in regulating invasion and metastasis of CRC.

Methods: The transcription factor and targets of *miR-214* were predicted by bioinformatics and validated using ChIP and dual-luciferase reporter assay. DNA methylation status was explored using bisulphite sequencing PCR. The *in vitro* and *in vivo* function of *miR-214* in CRC was evaluated using MTT, plate colony formation, Matrigel invasion and animal models. Real-time PCR or western blotting was performed to detect *FOXD3*, *miR-214* and *MED19* expressions in CRC cells and clinical specimens.

Results: *MiR-214* was downregulated in CRC and was significantly correlated with lymphatic metastasis. Downregulation of *miR-214* might due to promoter hypermethylation in CRC. *FOXD3* was validated as a transcription factor of *miR-214* by ChIP assay. Dual-luciferase assay identified *MED19* as a target of miR-214 in CRC. *In vitro* and *in vivo* experiments showed that *miR-214* mediated the inhibiting effect of *FOXD3* on proliferation, invasion and metastasis by targeting *MED19*. Spearman's correlation analysis showed a positive correlation between *FOXD3* and *miR-214*, and negative correlations between *FOXD3* and *MED19*, *miR-214* and *MED19* in CRC cells and clinical specimens.

Conclusions: *FOXD3*/*miR-214*/*MED19* axis is important for the regulation of growth, invasion and metastasis of CRC. Targeting the *miR-214*-mediated axis might be helpful for the treatment of CRC.

Colorectal cancer (CRC) is a common malignancy in the world, its incidence and mortality rise gradually (Torre *et al*, 2015). Considering its high mortality, elucidate the molecular mechanisms of CRC metastasis and provide theoretical and experimental basis for clinical treatment are of vital importance. MiRNAs are a non-protein coding class of short regulatory RNAs (22-nucleotides long) involved in the regulation of a variety of physiological and pathological progresses through post-transcriptionally modulating gene expression, such as tumour development and progression, cell proliferation, apoptosis and basal metabolism (Bueno *et al*, 2008; Nicoloso *et al*, 2009; Inui *et al*, 2010; Voorhoeve, 2010; Hao *et al*, 2014). Dynamin-3 gene (*DNM3*) is a member of Dynamin family that has a key role in endocytosis and possessing mechanochemical properties of tabulating and severing membranes (Zhang *et al*, 2016). *MiR-214* is located in the chromosomal region 1q24.3, 14th intron of DNM3 and encoded within DNM3 opposite strand and

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Received 15 June 2016; revised 4 October 2016; accepted 8 October 2016; published online 3 November 2016

has vital roles in the regulation of cancer onset, growth, and progression (Penna *et al*, 2015). *MiR-214* is downregulated in several human tumours including breast, cervical, rhabdomyosarcoma, and hepatocellular carcinomas (Derfoul *et al*, 2011; Shih *et al*, 2012; Huang *et al*, 2014; Wen *et al*, 2014). The pleiotropic and tumour-specific of *miR-214* contributes to various cancer formation and progression via its specific target genes. Moreover, *miR-214* is a critical component involved in many fundamental signalling pathways such as *PTEN*/*Akt*, *β-catenin*, and tyrosine kinase receptor pathways (Wang *et al*, 2012a, b; Momose *et al*, 2013). Interestingly, recent reports have identified that miR-214 alterations in tumour cells lead to negative regulation of CRC liver metastasis (Chen *et al*, 2014). On the basis of this, *miR-214* is considered to be a potential target for tumour diagnosis, treatment and prognosis.

In this study, we report the suppressive role of the *FOXD3*/*miR-214*/*MED19* axis in CRC cells. We provide evidence that *miR-214* induced by its upstream transcription factor *FOXD3*, can suppress tumour growth and metastasis in CRC by targeting *MED19*.

MATERIALS AND METHODS

Cell lines, human tissue samples, and animals. Human CRC cell lines LOVO, SW620, SW480, HCT116, HT-29, LS174T and human embryonal kidney 293 cells were purchased from Shanghai Cell Bank of Type Culture Collection. The cell lines were freshly authenticated in last year. The cell lines were cultured in DMEM medium (GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) in 5% CO_2 at 37 °C. Images of CRC cells were taken by Olympus inverted microscope and were outputted by CellSens Dimension software (Olympus, Shinjuku, Japan). Paired fresh CRC tissues were collected from 30 patients who underwent CRC resection without prior radiotherapy and chemotherapy in Nanfang Hospital in 2010. These samples were snap-frozen in liquid nitrogen immediately after resection, and then stored at −80 °C until needed. Four- to 6-week-old male athymic BALB/c-nu/nu mice were purchased from the Central Laboratory of Animal Science of Southern Medical University (Guangzhou, China), and maintained in a specific pathogen free environment. All protocols for animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Southern Medical University.

Construction of plasmids and transfection. Lentiviral constructs expressing *miR-214* (Lenti-miR microRNA precursor clone collection; System Biosciences, Carlsbad, CA, USA) were packaged using the pPACKH1 lenti-vector Packaging Kit (System Biosciences). ShRNAs towards *FOXD3* (System Biosciences) were cloned into pSuper-retro-puro. Lentiviral constructs were used to infect CRC cells to establish cells stably expressing *miR-214* and *MED19* or repressing *FOXD3*. In the rescue experiments, *FOXD3*-depleting cells were transfected with *miR-214* vector. *MiR-214* inhibitor and its negative control were antisense oligos obtained from Genechem Company (Shanghai, China), and was used to transfect indicated cells according to the manufacturer.

DNA methylation analysis. DNA methylation analysis of *DNM3* was performed as previously descriped (He *et al*, 2015). In brief, genomic DNAs of paired CRC tissues were obtained using Promega wizard genomic DNA purification kit (Promega, Salt Lake City, UT, USA) and then bisulfite-modified using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA). The CpG island of *DNM3* gene was predicted online UCSC Genome Bioinformatics (http://www.genome.ucsc.edu/). The primers used in bisulfite genomic-sequencing PCR (BSP) detection were designed as following (F: 5′-TTGTATATGTTTGATGTGGTTTTAG-3′; R: 5′-TTCCTCTAAAATAAATTCCATAATCC-3'). The PCR reaction was performed at 95 °C for 5 min, then 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, followed by an extra extension at 72 °C for 5 min. The BSP products were confirmed by electrophoresis on a 1% agarose gel. Finally, they were cloned into a pMD19-T (TaKaRa, Osaka, Japan), and sequenced (Taihegene Biotechnology Co Ltd, Beijing, China).

MTT, plate colony formation, cell invasion assays *in vitro*. The MTT, plate colony formation, cell invasion assays of transfected CRC cells were determined as previously described (Liang *et al*, 2013).

Animal models. To evaluate the *in vivo* tumorigenic effects, 4×10^6 cells were injected subcutaneously into the flank of nude mice ($n = 5$ per group). Tumour size was measured with calipers to estimate volume every 6–7 days until day 28 after injection. The mice were sacrificed and tumours were collected 28 days later. For tail vein metastasis assay, 4×10^6 cells were injected into the tail vein of nude mice. After 2 months, mice were sacrificed and various organs from the thoracic, peritoneal and retroperitoneal cavities were removed, rinsed, fixed and subjected to pathological examination. The number of tumour colonies was determined by using a dissecting microscope. All animal experiments were conducted in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Southern Medical University.

Luciferase activity assay. For luciferase reporter assays, the 3′ untranslated region (3′ UTR) segment or promoter of *MED19* gene was amplified by PCR and inserted into the vector. Co-transfections of *MED19* 3′ UTR plasmid with *miR-214* lentivirus vector or *MED19* promoter plasmid with *FOXD3* vector into indicated cells were accomplished by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For the binding of *FOXD3* to *miR-214* promoter or *MED19* promoter, the coding region of *FOXD3* and the 2 kb region directly upstream of *miR-214* or the 1.3 kb region directly upstream of *MED19* transcription binding site were amplified by PCR and then inserted into the vectors respectively. Luciferase activity was measured 48 hours after transfection by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Each assay was repeated in three independent experiments.

Chromatin immunoprecipitation (ChIP) assay. According to the ChIP Assay Kit (Millipore, Darmstadt, Germany) protocol, SW620 and HT-29 cells were lysed using SDS lysis buffer and DNA was sheared by sonication to lengths between 200 bp and 1000 bp. Protein-DNA complexes were precipitated by anti-*FODX3* (Abcam, Cambridge, MA, USA) and anti-IgG antibody respectively. Crosslinks in protein-DNA complexes were then reversed by NaCl. The immunoprecipitated DNA was amplified by PCR for specific sequences (R1) containing putative *FOXD3* binding sites.

Immunohistochemical staining (IHC). Four-micrometer-thick histology sections from xenograft tumours were cut, deparaffinised using xylene, and hydrated through graded alcohol to water. Antigen retrieval was performed by boiling at 100 °C for 10 min in 10 mmol/l citrate buffer (pH = 6.0). In brief, these sections were incubated in polyclonal antibody against human *Ki-67* (Abnova, Taiwan) overnight at 4 °C. Subsequently, the horseradish-peroxidase-conjugated anti-goat secondary antibody (DakoCytomation, Glostrup, Denmark) was applied and incubated for 1 h at room temperature. The visualisation signal was developed with 3, 3-diaminobenzidine tetra hydrochloride staining, and the slides were counterstained in hematoxylin.

Statistical analysis. All statistical analyses were performed using SPSS 13.0 statistical software and were calculated from 3 independent experiments. Statistical significance was determined using Student *t* test, Fisher's exact test, or one way analysis of variance (ANOVA) as

appropriate. Spearman's correlation coefficient was used to measure the degree of the linear relationship of gene expression levels. Growth curves were generated using the log-rank test. $P<0.05$ was considered to be statistically significant.

RESULTS

Identification of *FOXD3*/*miR-214*/*MED19* axis in CRC. In our previous study, a miRNA microarray assay was used to screen potential metastasis associated miRNAs in CRC tissues with lymphatic metastasis and those without lymphatic metastasis (Li *et al*, 2015). From the list of differentially expressed miRNAs, we focused on *miR-214* because it was one of the most downregulated miRNAs in CRC tissues with lymphatic metastasis and its underlying mechanisms in CRC metastasis remain unclear (Supplementary Figure S1A). Real-time PCR analysis showed that *miR-214* was significantly downregulated in 30 paired fresh CRC tissues compared to normal mucosa (Figure 1A). Meanwhile, *miR-214* expression was markedly lower in primary CRC tissues with

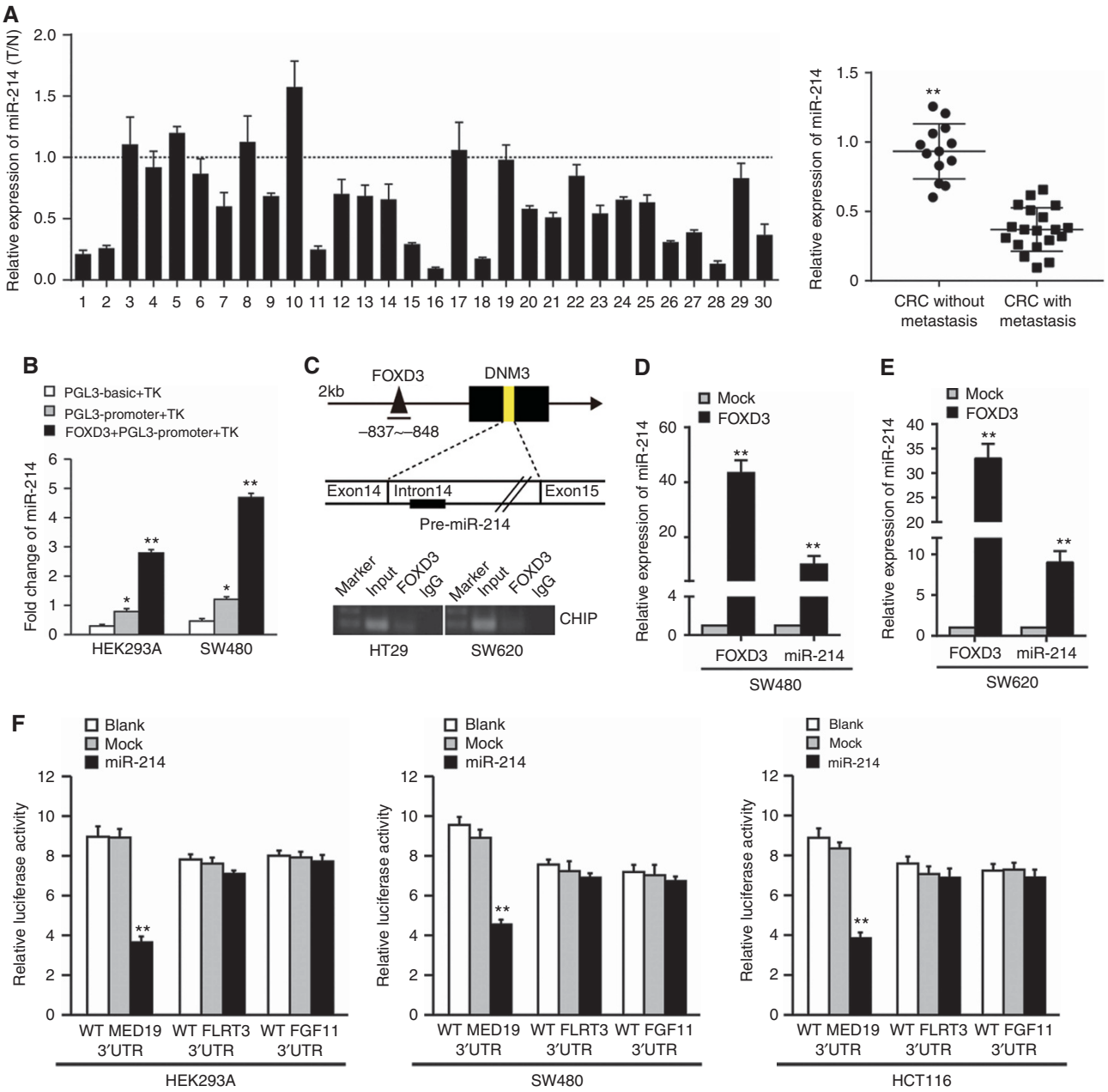

Figure 1. Identification of a *FOXD3*/*miR-214*/*MED19* axis in human colorectal cancer. (**A**) Real-time PCR analysis of *miR-214* expression in 30 paired cases of the primary CRC tissues with or without metastasis and matched adjacent normal mucosa. The expression of *miR-214* in normal mucosa were normalised to 1. (**B**) Luciferase activity of PGL3-*miR-214*-promoter construct after transfection of *FOXD3* plasmid in HEK293A and SW480 cells. (**C**) Consite and TFsearch predicted the 2000 bp upstream region of *miR-214* for putative transcriptional factors binding site. Chromatin immunoprecipitation assay was performed in HT-29 and SW620 cells transfected with *FOXD3*-expressing vector. (**D** and **E**) Real-time PCR analysis of *FOXD3* expression in *FOXD3* overexpressing SW480 and SW620 cells. (**F**) Luciferase activities of wild-type 3′-UTR-*MED19*-luc, 3′UTR-*FLRT3*-luc and 3′UTR-*FGF11*-luc constructs in HEK293A, SW480 and HCT116 cells after transfection of *miR-214* plasmid. $^{*}P<0.05$, $^{**}P<0.01$. Data represent the mean ± SD.

lymphatic metastasis than those without lymphatic metastasis (Figure 1A). These results indicate that *miR-214* is downregulated in CRC, which might be associated with CRC metastasis.

To investigate the potential molecular mechanism of *miR-214* in CRC, we analysed transcription factors that are located within 2 kb region directly upstream of the transcription start site of *miR-214*

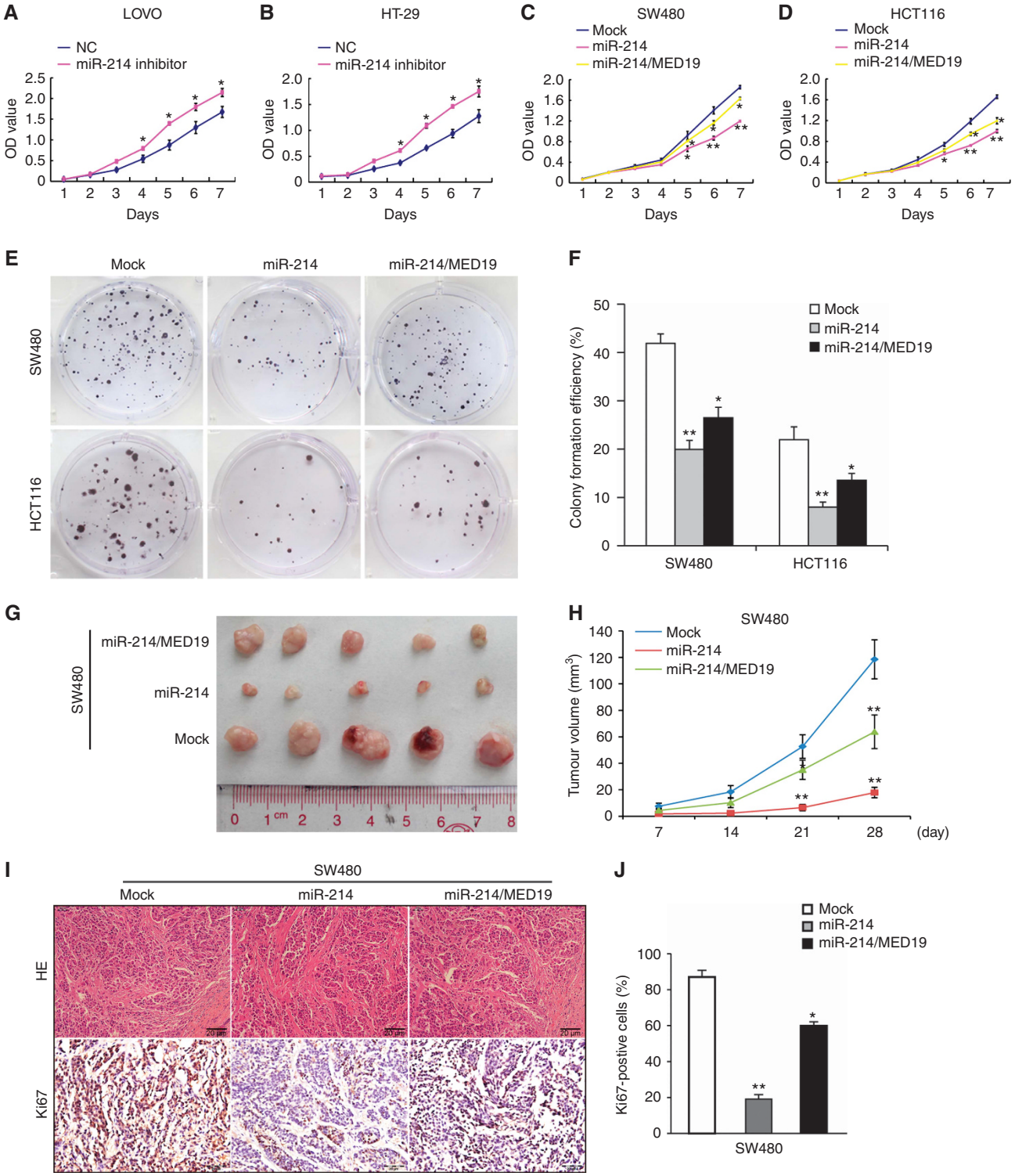

Figure 2. *miR-214* suppresses tumour proliferation by target gene *MED19* in CRC. (**A** and **B**) Effect of *miR-214* inhibitor on proliferation of LOVO and HT-29 cells by MTT assay. (**C** and **D**) Effects of *miR-214* and *miR-214/MED19* on proliferation in SW480 and HCT116 cells by MTT assay. (**E** and **F**) Effects of *miR-214* and *miR-214/MED19* on cell proliferation in SW480 and HCT116 cells by colony formation assay. (**G** and **H**) Subcutaneous tumours of nude mice injected with SW480/Mock, SW480/*miR-214* and SW480/*miR-214/MED19* cells. The nodules volume of subcutaneous tumours was measured. (**I** and **J**) Hematoxylin–eosin (HE) staining and immunohistochemical (IHC) staining of *Ki-67* expression in subcutaneous tumours of nude mice injected with SW480/Mock, SW480/*miR-214* and SW480/*miR-214/MED19* cells. *Ki-67* positive expression rate was measured. Scare bars represent 20 μm. $^{*}P<0.05$, $^{**}P<0.01$. Data represent the mean ± SD.

using Consite and TFsearch databases (Supplementary Figure S1B). One binding motif was found for *FOXD3* within −837 bp to −848 bp regions in the promoter of *miR-214*. Luciferase reporter assay showed that *FOXD3* effectively elevated the luciferase activity of *miR-214* promoter in HEK293A and SW480 cells (Figure 1B). ChIP assay was performed to further investigate the combination of *FOXD3* with *miR-214* promoter. Results showed that *FOXD3* could directly bind the region of −837 bp to −848 bp in the promoter of *miR-214* in HT-29 and SW620 cells (Figure 1C). After that, we analysed the expression of *miR-214* in *FOXD3*-expressing SW480 and SW620 cells. *FOXD3* markedly increased the expression of *miR-214* (Figures 1D–E). These data suggest that *FOXD3* binds to specific promoter of *miR-214* and activates transcription.

MicroRNAs suppress gene expression through interacting with the 3′ UTRs of target mRNAs (Ioshikhes *et al*, 2007). To identify the downstream targets of *miR-214*, we used five miRNA target-predicting databases including miRanda, TargetScan, Picta, RNAhybrid, miRBase. *MED19*, *FLRT3*, and *FGF11* were selected as potential targets due to their overlap among all databases and metastasis-related functions (Hu *et al*, 2007; Chen *et al*, 2009; Wen *et al*, 2013). We cloned the 3' UTRs of *MED19*, *FLRT3* and *FGF11* into a luciferase plasmid psiCHEK^{TM}-2 in HEK293A, SW480 and HCT116 cells. Among all cell lines, only the luciferase activity of 3' UTR of *MED19* was significantly suppressed by *miR-214* (Figure 1F). To determine whether *MED19* is transcriptionally regulated by *FOXD3*, *MED19* was ectopically overexpressed using plasmid of *MED19* promoter. We found that dual-luciferase activity of *MED19* promoter was not significantly affected by *FOXD3* compared to mock group (Supplementary Figure S1C). These above data indicate that transcription factor *FOXD3* induces *miR-214* transcription, subsequently down-regulates *MED19* expression, which constitutes a potential *FOXD3*/*miR-214*/*MED19* axis to play a role in CRC cells.

***MiR-214*-mediated suppression of *MED19* inhibits cell proliferation of CRC.** Gain-of-function and loss-of-function assays were performed to confirm whether *miR-214* functionally regulating the proliferation of CRC cells by targeting *MED19*. According to endogenous expression of *miR-214* in six CRC cell lines

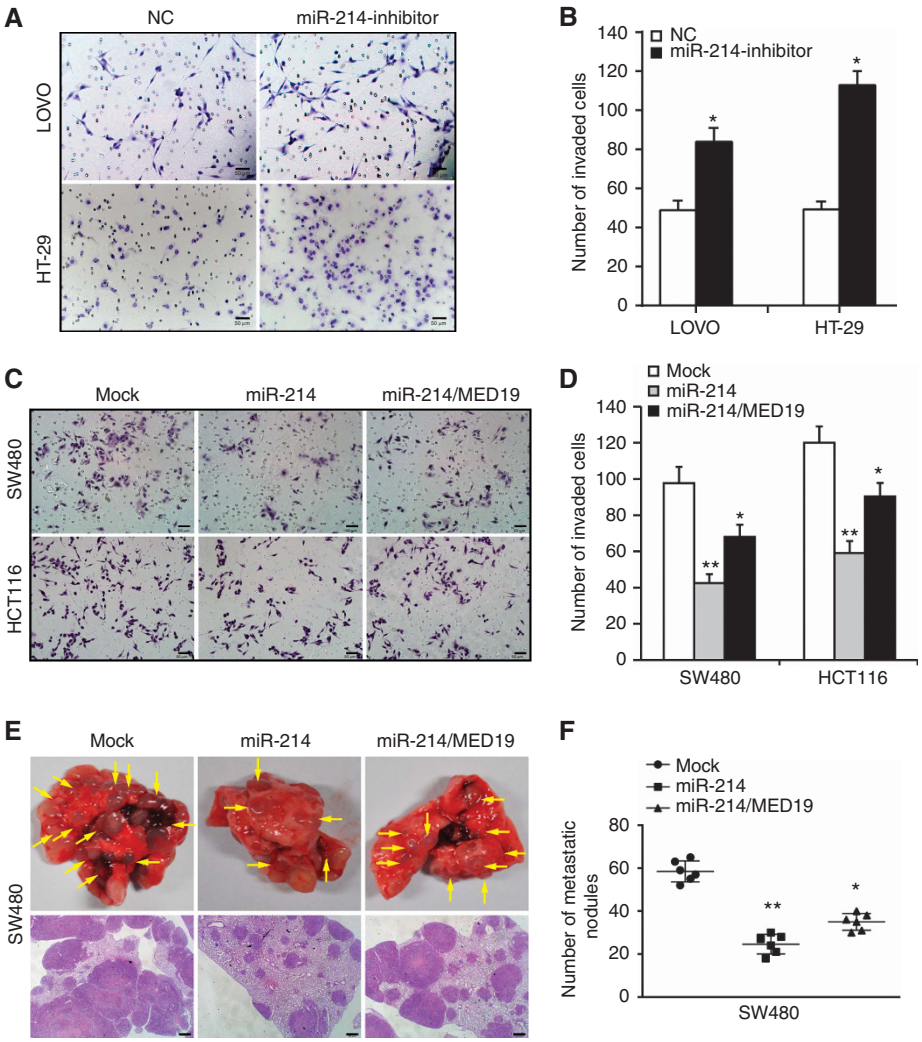

Figure 3. *miR-214* suppresses invasion and metastasis of CRC by targeting *MED19*. (**A** and **B**) Effect of *miR-214* inhibitor on the invasion of LOVO and HT-29 cells by Boyden chamber. Morphologic comparison of cells penetrating the artificial basement membrane was shown. Scale bars represent 50 μm. (**C** and **D**) Effects of *miR-214* and *miR-214*/*MED19* on cell invasion of SW480 and HCT116 cells by Boyden chamber. Morphologic comparison of cells penetrating the artificial basement membrane was shown. Scale bars represent 50 μm. (**E**) SW480/*Mock*, SW480/*miR-214* and SW480/*miR-214*/*MED19* cells were injected into the tail vein of nude mice. Yellow arrows in top panels point at lung metastatic nodules. Scale bars in bottom panels represent 500 μm. (**F**) The number of lung metastatic nodules per mouse was counted under the microscope. *$P<0.05$, **$P<0.01$. Data represent the mean ± SD.

(Supplementary Figure S1D), we transfected *miR-214*-expressing lentivirus vector into SW480 and HCT116 cells (Supplementary Figure S1E) and knocked down *miR-214* with inhibitor in LOVO and HT-29 cells (Supplementary Figure S1F). To perform the rescue experiments, we transfected a construct that encodes entire *MED19* coding domains, but lacking the 3′ UTR region into *miR-214*-expressing cells. Reintroduction of *MED19* rescued *MED19* expression in *miR-214*-expressing cells, but did not change the expression of *miR-214* (Supplementary Figure S1G–H). MTT assay showed that *miR-214* inhibitor significantly increased the growth rate of LOVO and HT-29 cells compared with control group (Figure 2A–B). On the contrary, ectopic *miR-214* expression inhibited the proliferation of SW480 and HCT116 cells (Figure 2C–D). Consistently, *miR-214* obviously suppressed colony formation of SW480 and HCT116 cells (Figure 2E–F). MTT assay, Boyden chamber, and Matrigel invasion assay showed that ectopic *MED19* expression could significantly promote SW480 and LOVO cell proliferation, migration and invasion *in vitro* (Supplementary Figure S2A–C). Constitutive *MED19* expression rescued the proliferation and colony formation phenotypes in *miR-214*-expressing cells (Figure 2C–F).

To further investigate the effect of *miR-214* on cell proliferation *in vivo*, we subcutaneously injected SW480/*miR-214*, SW480/*miR-214*/*MED19* and control cells into nude mice. Subcutaneous tumour volume in *miR-214* group was significantly decreased compared with mock group, whereas co-expression of *MED19* reversed the suppression induced by *miR-214* (Figure 2G–H). IHC analysis showed that *Ki-67* expression in *miR-214*-expressing tumours was lower than that in control group, while reintroduction of *MED19* displayed increased *Ki-67* index (Figure 2I–J, Supplementary Figure S3A–B). Our results thus reveal that *miR-214* inhibits the proliferation of CRC cells by targeting *MED19*.

***MiR-214*-mediated suppression of *MED19* inhibits invasion and metastasis of CRC.** We also assessed the effect of *miR-214* on invasion and metastasis of CRC cells. Matrigel invasion assay showed that *miR-214* knockdown in LOVO and HT-29 cells obviously increased the number of invaded cells (Figure 3A and B), whereas ectopic *miR-214* showed the opposite effect in SW480 and HCT116 cells (Figure 3C and D). Reintroduction of *MED19* could partly reverse the suppression of *miR-214* on invasion (Figure 3C and D). To test the effect of *miR-214* on CRC metastasis, SW480/*miR-214* cells, SW480/*miR-214*/*MED19* cells and control cells were injected into tail vein of nude mice. The lung metastatic rate of SW480/mock, SW480/*miR-214*, and SW480/*miR-214*/*MED19* group in nude mice was 100% (6/6), 33.33% (2/6), and 66.67% (4/6), respectively. Large lung metastatic nodules could be detected in SW480/*miR-214*/*MED19* and SW480/mock groups, while only few small nodules were observed in SW480/*miR-214* group (Figure 3E). Moreover, the number of metastatic nodules was obviously reduced in mice injected with SW480/*miR-214* cells compared to mock cells, whereas *MED19* and *miR-214* co-expressing cells caused increased metastatic nodules compared to SW480/*miR-214*-expressing cells (Figure 3F). Therefore, these results demonstrate that *miR-214* suppresses invasion and metastasis of CRC via downregulation of *MED19*.

Promoter hypermethylation might contribute to the transcriptional silencing of *miR-214* in CRC. Recent studies indicate that miRNA expression can be deregulated in cancer by different epigenetic mechanisms, including aberrant methylation of the promoter regions or histone modifications (Scott *et al*, 2006; Suzuki *et al*, 2013). DNA methylation of promoter associated CpG islands has been reported for several microRNAs, such as *miR-137* (Bier *et al*, 2013); *miR-145* (Lee *et al*, 2013), *miR-204* (Ying *et al*, 2013). Interestingly, *miR-214* was an intronic miRNA located between exons 14 and 15 of *DNM3* gene, so we investigated the methylation status of *DNM3* promoter. Gene Expression Omnibus (GEO) database was used to analyse the CpG island methylation of DNM3 promoter and results indicated that the methylation status of DNM3 promoter in CRC was higher than in normal mucosa (Figure 4A). We next analysed the methylation status of DNM3

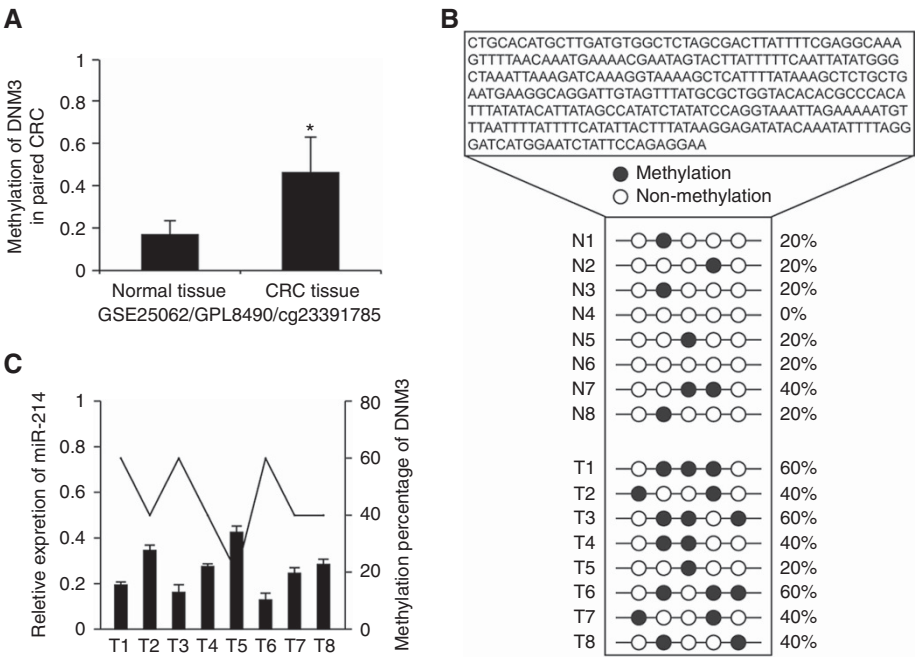

Figure 4. Promoter hypermethylation contributes to the transcriptional silencing of *miR-214* in CRC. (**A**) The analysis results of GEO showed that the degree of CpG Island methylation in CRC was higher than that in normal mucosa. (**B**) Bisulphite genomic-sequencing analysis of the CpG island of DNM3 promoter in paired CRC tissue. (**C**) The relationship between the expression of *miR-214* and the degree of CpG Island methylation in eight cases of CRC tissues and corresponding normal mucosa. *miR-214* expression was determined by real-time PCR. The methylation percentage was calculated using the BSP results from B. $*P<0.05$, $**P<0.01$. Data represent the mean ± SD.

promoter in eight cases of paired CRC tissues by BSP. BSP results showed increased CpG island methylation of *DNM3* promoter in CRC tissues (Figure 4B). Combining the results of real-time PCR and BSP, we found that *miR-214* expression was decreased in CRC tissues with hypermethylation status of *DNM3* promoter (Figure 4C). These results indicate, to a certain extent, that hypermethylation of DNM3 promoter might lead to transcriptional silence of *miR-214* in CRC.

***FOXD3* upregulates *miR-214* transcription and suppresses CRC cell proliferation *in vitro* and *in vivo*.** To further investigate whether *miR-214* affects the function of *FOXD3* in the progression of CRC, we silenced *FOXD3* with three siRNAs in SW480 and SW620 cells according to endogenous expression of *FOXD3* in six CRC cell lines. According to transfection efficiency, we chose siRNA3 to perform following experiment (Supplementary Figure S3C and D). Subsequently, we established stable *FOXD3*-depleting SW480 and SW620 cells and then stably transfected *FOXD3*-depleting cells with *miR-214* and confirmed its over-expression (Supplementary Figure S3E). Compared with NC group, knockdown of *FOXD3* promoted cell proliferation and colony formation *in vitro*, while reintroduction of *miR-214* incompletely reversed the promotion (Figures 5A and D). Moreover, ectopic *miR-214* expression reduced the expression of *MED19*. Knockdown of *FOXD3* led to increased expression of *MED19*, whereas enhanced expression of *miR-214* abolished *FOXD3* responsiveness (Figure 5E).

We further examined the effect of *FOXD3* on tumour growth *in vivo*. SW620/*shFOXD3*/*miR-214*, SW620/*shFOXD3* and control cells were subcutaneously injected to nude mice respectively. Mice injected with SW620/*shFOXD3* cells had increased subcutaneous tumour volume compared with those injected with SW620/NC cells, whereas constitutive *miR-214* rescued tumour growth induced by *FOXD3* knockdown (Figures 5F–G). Immunohistochemistry staining exhibited higher positive rate of *Ki-67* index in *FOXD3*-depleting tumour than control group, whereas *miR-214* could partly abrogated the *Ki-67* expression induced by *FOXD3* knockdown (Figures 5H and I, Supplementary Figure S3F and G). These results indicate that *FOXD3* upregulates *miR-214* transcription, then suppresses the proliferation of CRC cells.

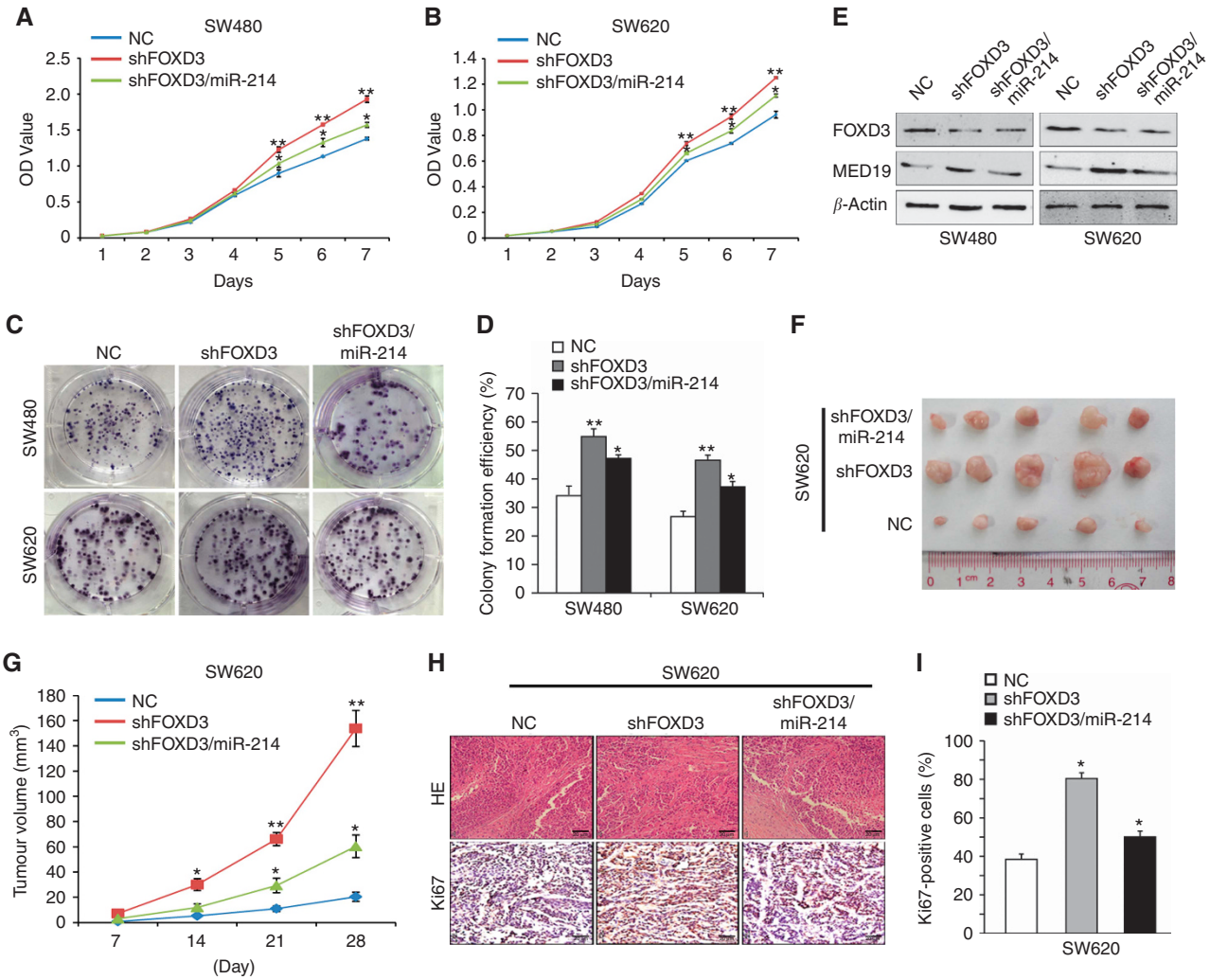

Figure 5. *FOXD3* upregulates *miR-214* expression and suppresses tumorigenesis. (**A**, **B**) Effects of *shFOXD3* and *shFOXD3/miR-214* on cell proliferation in SW480 and SW620 cells by MTT assay. (**C**, **D**) Effects of *shFOXD3* and *shFOXD3/miR-214* on cell proliferation in SW480 and SW620 cells by colony formation assay. (**E**) *MED19* expression in cells treated with NC, *shFOXD3* or *shFOXD3/miR-214* by Western blot. Expression levels were normalised to *β-actin*. (**F**, **G**) Subcutaneous tumours of nude mice injected with SW620/*NC*, SW620/*shFOXD3* and SW620/*shFOXD3/miR-214* cells. The nodules volume of subcutaneous tumours was measured. (**H**, **I**) Hematoxylin–eosin(HE) staining and immunohistochemical (IHC) staining of *Ki-67* expression in subcutaneous tumours of nude mice injected with SW620/NC, SW620/*shFOXD3* and SW620/*shFOXD3/miR-214* cells. *Ki-67* positive expression rate was measured. Scale bars represent 20 μm. *$P<0.05$, **$P<0.01$. Data represent the mean ± SD.

***FOXD3* upregulates *miR-214* transcription and suppresses CRC invasion and metastasis *in vitro* and *in vivo*.** We next investigated the role of *FOXD3* in CRC invasiveness and metastases. Results of Matrigel invasion assay showed that knockdown of *FOXD3* enhanced invasiveness of CRC cells *in vitro*, while reintroduction of *miR-214* attenuated the promoting effects of *FOXD3* knockdown (Figures 6A–B). To confirm whether downregulation of *FOXD3* is associated with CRC metastasis *in vivo*, SW620/NC, SW620/*shFOXD3* and SW620/*shFOXD3*/*miR-214* cells were injected into tail vein to seed lung metastases. In the group of mice injected with SW620/NC cells, only 16.667% (1 of 6) of mice had lung metastases. However, lung metastatic rate of mice injected with SW620/*shFOXD3* and SW620/*shFOXD3*/*miR-214* was 83.333% and 50%, respectively (Figure 6C). The number and volume of metastatic nodules in the lung were significantly increased in mice injected with *FOXD3*-depleting cells compared with those injected with control cells. Moreover, less lung metastatic nodules were observed in *shFOXD3*/*miR-214* group than *shFOXD3* group (Figure 6D). On the basis of these results, it would be reasonable to conclude that *FOXD3* suppresses invasion and metastasis of CRC by upregulating *miR-214*.

Correlations of *miR-214* with *MED19*, *FOXD3* expressions in CRC cell lines and clinical specimens. To demonstrate the relationship between *miR-214*, *MED19* and *FOXD3* expressions in CRC cell lines and tissues, we detected expressions of *miR-214*, *MED19* and *FOXD3* in 6 CRC cell lines and 18 paired cases of human CRC clinical specimens. Spearman's correlation analyses showed a positive correlation between *FOXD3* and *miR-214*, and negative correlations between *miR-214* and *MED19*, *FOXD3* and *MED19* expressions in six CRC cell lines (Figures 7A and B). In addition, *miR-214* was obviously downregulated, whereas *MED19* was upregulated in CRC clinical specimens compared with corresponding normal mucosa (Figures 7C and D). Spearman's correlation analyses showed a positive correlation between *FOXD3* and *miR-214* and negative correlations between *miR-214* and *MED19*, *FOXD3* and *MED19* expressions (Figure 7E).

DISCUSSION

Increasing evidence has highlighted that miRNAs are aberrantly expressed or mutated in human cancer, indicating that they may function as a novel class of oncogenes or tumour suppressor genes (Calin *et al*, 2002; Ma *et al*, 2013); Chan and Wang, 2015. For instance, *miR-204* suppressed self-renewal, stem-cell-associated phenotype, and migration of glioma cells by targeting for *SOX4* and *EphB2* (Ying *et al*, 2013); *miR-21* was markedly elevated in human glioblastoma tumour and contribute to the maliganant phenotype by blocking expression of critical apoptosis-related genes (Chan *et al*, 2005). In our previous research, a list of deregulated miRNAs were screened out using miRNA microarray, including *miR-137*, *miR-371-5p* and *miR-214*. *MiR-137* was able to suppress CRC invasion and metastasis by way of regulating *FMNL2* (Liang *et al*, 2013); *miR-371-5p* inhibited EMT, stem cell properties and metastasis by targeting *SOX2* in CRC (Li *et al*, 2015). *MiR-214*, located in the 14th intron of *DNM3*, was another significantly deregulated miRNA we found in CRC specimens. Emerging studies showed that *miR-214* was implicated in many human physiological and pathological process. For instance, Li *et al* (2016) found that increased osteoclastic *miR-214-3p* reduced bone formation in elderly woman with fractures and in ovariectomised mice (Li *et al*, 2016); *miR-214* was decreased

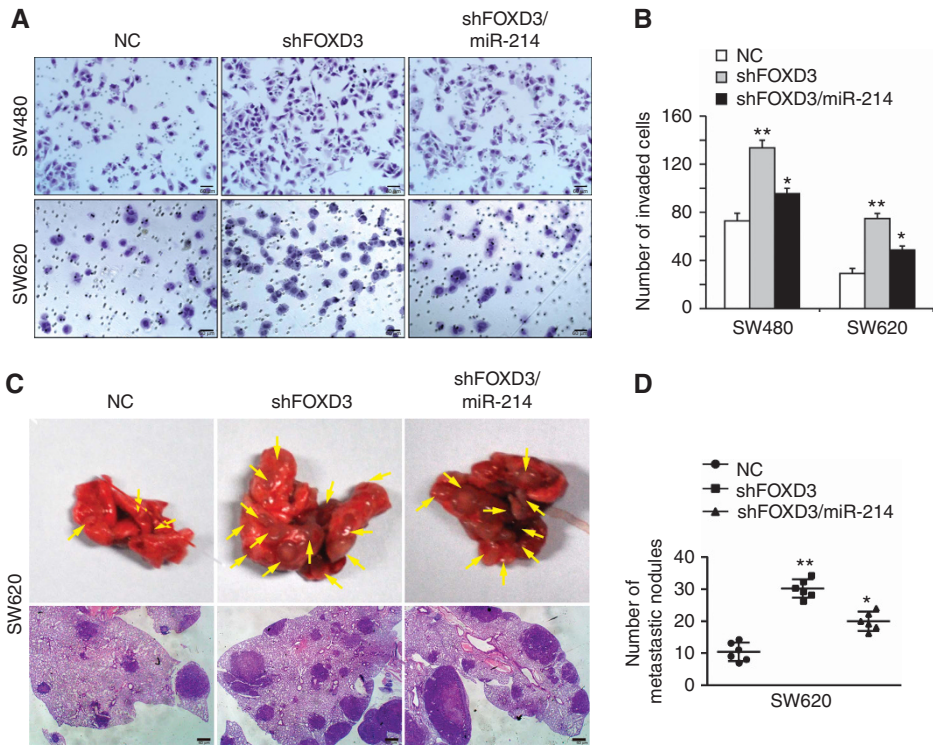

Figure 6. *FOXD3* upregulates *miR-214* expression and suppresses CRC invasion and metastasis. (**A**) Effects of *shFOXD3* and *shFOXD3*/*miR-214* on cell invasion of SW480 and SW620 cells by Boyden chamber. Morphologic comparison of cells penetrating the artificial basement membrane was shown. Scale bars represent 50 μm. (**B**) The number of invaded SW480 and SW620 cells was measured under the microscope. (**C**) SW620/NC, SW620/*shFOXD3* and SW620/*shFOXD3*/*miR-214* cells were injected into the tail vein of nude mice. Yellow arrows in top panels point at lung metastatic nodules. Scale bars in bottom panels represent 500 μm. (**D**) The number of lung metastatic nodules per mouse was counted under the microscope. *$P<0.05$, **$P<0.01$. Data represent the mean ± SD.

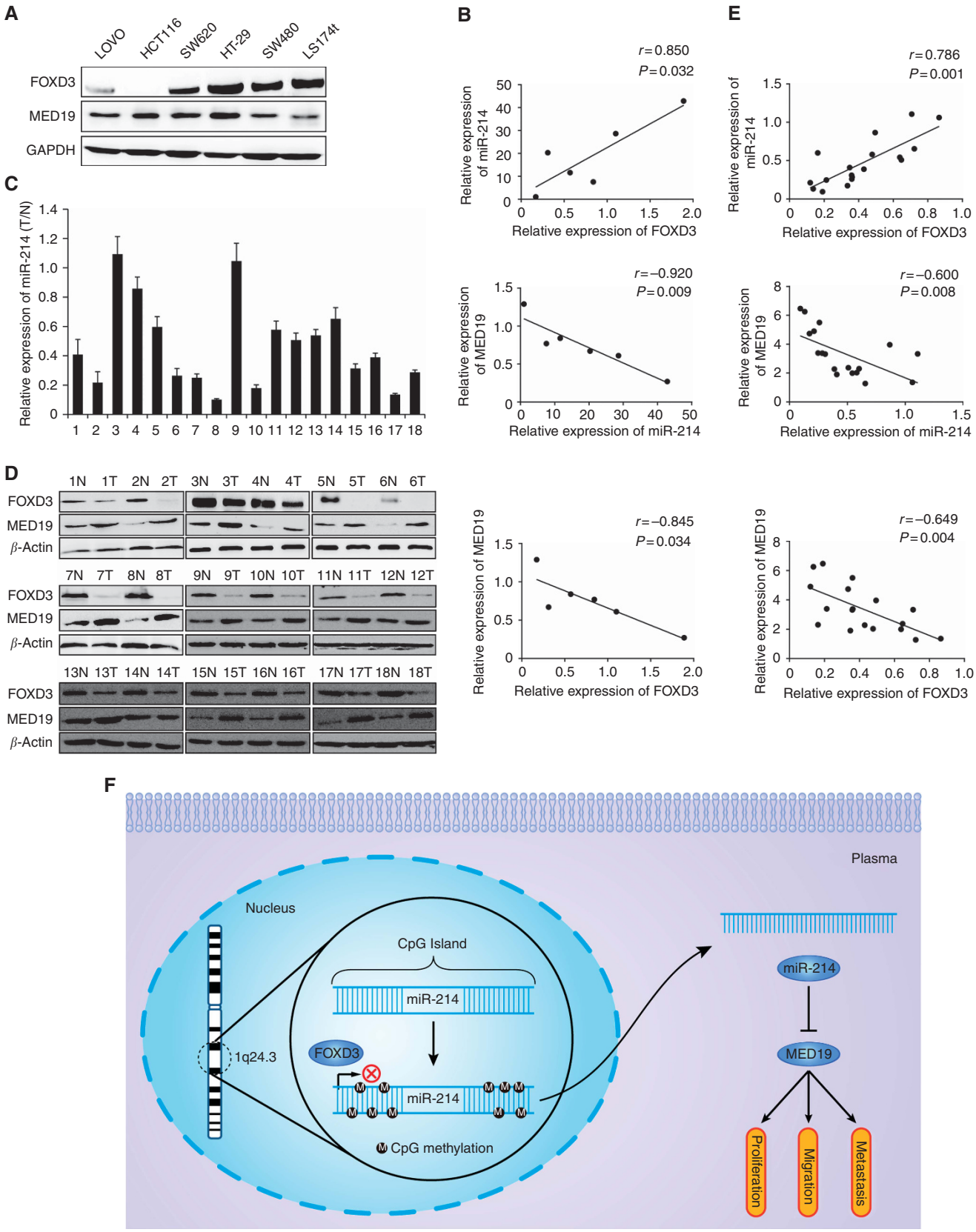

Figure 7. Correlations of *miR-214* with *MED19*, *FOXD3* expression in CRC cell lines and tissues. (**A**) Western blot analysis of endogenous expression of *FOXD3* and *MED19* in six CRC cell lines. Expression levels were normalised to *GAPDH*. (**B**) Spearman's correlation analysis of the expression between *FOXD3* and *miR-214*, *miR-214* and *MED19*, *FOXD3* and *MED19* in six CRC cell lines. (**C**) Real-time PCR analysis of *miR-214* expression in 18 paired primary CRC tissues (T) and matched adjacent normal mucosa (N). (**D**) Western blot analysis of *FOXD3* and *MED19* expression level in 18 paired primary CRC tissues (T) and corresponding normal mucosa (N). Expression levels were normalised to *β-actin*. (**E**) Spearman's correlation analysis of the expression between *FOXD3* and *miR-214*, *miR-214* and *MED19*, *FOXD3* and *MED19* in 18 paired CRC tissues. (**F**) Schematic diagram of *FOXD3/miR-214/MED19* axis in the regulation of proliferation, migration and metastasis of human colorectal cancer.

significantly in Parkinson's disease (PD) patients and may represent a novel biomarker for the early detection of PD (Dong *et al*, 2016); *miR-214* might promote Th17 cell differentiation by targeting *mTOR* signalling in purified CD4+ T cells of multiple sclerosis (Ahmadian-Elmi *et al*, 2016); Sun *et al* (2015) found that *miR-214* mediated CF proliferation and collagen synthesis via inhibition of *Mfn2* and activation of *ERK1/2 MAPK* signalling . In addition, *miR-214* has been reported to be deregulated in many human tumours. Owing to 'oncomir' or 'tumour suppressor-mir' functions of miRNA in different tumour, it is still urged to investigate its potential regulatory molecular mechanisms for gaining better clinic diagnosis and treatment of CRC. In the present study, we set out to explore the role of *miR-214 in vitro* and *in vivo* and put forward a *FOXD3*/*miR-214*/*MED19* axis involved in cell proliferation, migration and metastasis of CRC.

MiR-214 expression was apparently downregulated in CRC clinical specimens by real-time PCR, which was consistent with the microarray assay, and its downregulation was associated with CRC lymphatic metastasis. What is more, we found a hypermethylation status of promoter region in *miR-214*-encoding gene *DNM3* by BSP, and this might explain the downregulation mechanism of *miR-214* in CRC. Expression of *miR-214* is deregulated in many tumours including pancreatic cancer, melanoma, and hepatocellular carcinoma (Zhang *et al*, 2010; Penna *et al*, 2013; Zhang *et al*, 2015), whereas upregulated in breast cancer by targeting p53 (Wang *et al*, 2015). The pleiotropic and tumour-specific of *miR-214* contributes to various cancer formation and progression via its several target genes including *p53*, *Bcl-2/Bax*, *TFAM*, *EZH2* (Wen *et al*, 2014; Yang *et al*, 2014; Tian *et al*, 2015). Recently, Chen *et al* (2014) reported that *miR-214* negatively regulated liver metastasis in CRC . However, the molecular mechanism of *miR-214* in CRC metastasis has still not been illustrated. Therefore, we explored potential *miR-214*-mediated mechanism in the progression of CRC. Generally, miRNAs showed to be regulated by the upstream transcription factors (O'Donnell *et al*, 2005). We analysed the promoter region of *miR-214* and found *FOXD3* transcriptionally regulated *miR-214*. *FOXD3* has been reported to inhibit growth, invasion, metastasis and angiogenesis in several tumours (Li *et al*, 2013; Chu *et al*, 2014; Liu *et al*, 2014). Recently, van Roon *et al* (2013) found that *FOXD3* gene expression was repressed due to hypermethylation of promoter in human CRC. We speculate that hypermethylation of *FOXD3* gene could lead to its low expression in CRC, and this might subsequently cause low *miR-214* expression. Combined with bioinformatics search and dual-luciferase assay, *MED19* was found to be a major downstream effector of *miR-214*. Meanwhile, increasing evidence has revealed *MED19* as functional target involved in tumour progression (Li *et al*, 2011). Our results revealed that *FOXD3* inhibited the expression of *MED19* by upregulating the expression of *miR-214* in CRC cells. Thus, the *FOXD3*/*miR-214*/*MED19* axis might act as a key pathway in CRC metastasis.

Next, we identify that *miR-214*, induced by its upstream transcription factor *FOXD3*, can suppress tumour growth and metastasis in CRC by targeting *MED19*. The *in vitro* and *in vivo* '*MED19* rescue' experiments proved that *miR-214* suppressed tumour growth and metastasis in CRC mainly by targeting *MED19*. Mediator complex subunit 19 (*MED19*) is a member of the mediator that has a key role in the activation and repression of signal transduction or the regulation of transcription in carcinomas (Casamassimi and Napoli, 2007; Sun *et al*, 2011). Accumulating evidence has shown that *MED19* has important roles in cancer cell proliferation and tumorigenesis, and suppression of *MED19* expression induces inhibition of cell proliferation and tumorigenesis in several different tumour types including lung cancer, pancreatic cancer, ovarian cancer and breast cancer (Li *et al*, 2011; Liu *et al*, 2012; Wei *et al*, 2015). Then we speculated that *miR-214* played tumorigenic role by its target *MED19* in CRC. Further research confirmed that *FOXD3* stimulated the transcription activity of *miR-214*, subsequently significantly suppressed cell proliferation and metastasis by downregulation of *MED19* in CRC. *FOXD3* as a novel tumour suppressor has been reported highly connected with carcinogenesis (Chu *et al*, 2014). Hypermethylation of *FOXD3* suppresses cell proliferation, invasion and metastasis in hepatocellular carcinoma (He *et al*, 2015). And recent study also suggested that decreased *FOXD3* expression is associated with poor prognosis in patients with high-grade tumour (Du *et al*, 2015). Therefore, we provide evidence that the involvement of *FOXD3*/*miR-214*/*MED19* axis in tumour growth and metastasis of CRC.

Eventually, we detected the correlations of *miR-214*, *FOXD3* and *MED19* in six CRC cell lines and 18 paired cases of human CRC tissues. Our results showed that there was a positive relationship between the expression level of *miR-214* and *FOXD3*, and a negative relationship between *miR-214* and *MED19*, *FOXD3* and *MED19*. On the basis of these evidence, we clearly validate that *FOXD3* induces *miR-214* expression and consequently represses its target *MED19* in CRC.

In summary, *FOXD3*/*miR-214*/*MED19* axis has an important role in the regulation of CRC progression. Hypermethylation of *DNM3* promoter leads to low expression of *miR-214* in CRC. Function experiments demonstrate that *miR-214* mediates the suppressive role of *FOXD3* in proliferation, invasion and metastasis of CRC by targeting *MED19* (Figure 7F). The *FOXD3*/*miR-214*/*MED19* signalling axis might offer a promising therapeutic target for CRC treatment.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Program of China (973 Program, 2015CB554002), Key project of National Natural Science Fund (Guangdong Province NSFC- joint fund, U1201226), National Natural Science Foundation of China (81272759, 81172382, 81472313, 81401927); Natural Science Foundation of Guangdong Province (S2013010014544). We thank Professor Reddy for editing the English writing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GYH, JLH, LZ, XHZ, SNX, DZ, GFL, WTL carried out experiments. YQD and LL conceived experiments and analysed the data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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