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# MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis

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## Abstract

**Objective—**Distant metastasis is the major cause of cancer-related death in patients with colorectal cancer (CRC). Although the microRNA-200 (miR-200) family is a crucial inhibitor of epithelial-to-mesenchymal transition (EMT) in human cancer, the role of miR-200 members in the pathogenesis of metastatic CRC has not been investigated.

**Design—**Fifty-four pairs of primary CRC and corresponding matched liver metastasis tissue specimens were analysed for expression and methylation status of the miR-200 family members. Functional analysis of miR-200c overexpression was investigated in CRC cell lines, and cells were analysed for proliferation, invasion and migration. Expression of several miR-200c target genes (*ZEB1, ETS1* and *FLT1*) and EMT markers (E-cadherin and vimentin) in CRC cell lines and tissue specimens was validated.

**Results—**Liver metastasis tissues showed higher expression of miR-200c (primary CRC=1.31 vs. liver metastasis=1.59; p=0.0014) and miR-141 (primary CRC=0.14 vs. liver metastasis=0.17; p=0.0234) than did primary CRCs, which was significantly associated with hypomethylation of the promoter region of these miRNAs (primary CRC=61.2% vs. liver metastasis=46.7%; p<0.0001). The invasive front in primary CRC tissues revealed low miR-200c expression by in situ hybridization analysis. Transfection of miR-200c precursors resulted in enhanced cell proliferation but reduced invasion and migration behaviours in CRC cell lines. Overexpression of miR-200c in CRC cell lines caused reduced expression of putative gene targets, and resulted in increased E-cadherin and reduced vimentin expression. The associations between miR-200c,

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target genes and EMT markers were validated in primary CRCs and matching liver metastasis tissues.

**Conclusions—**miR-200c plays an important role in mediating EMT and metastatic behaviour in the colon. Its expression is epigenetically regulated, and miR-200c may serve as a potential diagnostic marker and therapeutic target for patients with CRC.

## INTRODUCTION

Distant metastasis is the major cause of death in patients with colorectal cancer (CRC). In spite of this, there is a paucity of effective treatments for managing patients with metastatic CRC. Consequently, a better understanding of the molecular mechanisms underlying distant metastasis is required to facilitate the development of effective therapeutic strategies for patients with metastatic CRC, or to prevent incipient metastasis in the adjuvant setting. One of the key molecular steps in the process of distant metastasis includes epithelial-to-mesenchymal transition (EMT), which permits invasion and emigration in various cancers,[12] and is associated with a poor prognosis in CRC.[3] EMT is a complex process, which includes dissolution of cell–cell junctions and loss of apicobasolateral polarity, resulting in the formation of migratory mesenchymal cells with invasive properties. During the EMT process, cancer cells lose the expression of cellular adhesion proteins such as E-cadherin and catenin, and acquire expression of mesenchymal markers such as vimentin and N-cadherin. Loss of E-cadherin expression is generally accepted as a hallmark of the EMT process, and various transcription factors that can regulate E-cadherin transcription are a matter of active investigation. E-cadherin repressors are generally classified into two groups: `direct repressors', such as SNAIL, ZEB1, ZEB2 and E47, which can directly repress E-cadherin gene transcription[4]; and `indirect repressors', such as Twist, Goosecoid, FoxC2 and E2.2, which downregulate E-cadherin transcription indirectly.[15]

Epithelial cancer cells that acquire migratory and invasive mesenchymal cell-like properties during EMT gain the ability to emigrate from the primary tumour mass and move to distant locations. Once metastasised, these cells require mesenchymal-to-epithelial transition (MET), a process that is the reverse of EMT, to facilitate the subsequent settlement and proliferation of disseminated cancer cells at secondary locations.[6] MET has been previously associated with cancer metastasis, and, in support of this, it has been demonstrated that liver-metastasised human prostate carcinoma cells express abundant E-cadherin, but lack vimentin expression.[7] Similarly, metastasised human breast carcinoma tissues have stronger E-cadherin expression than the corresponding primary tumour.[8]

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression. It has suggested that the expression of up to 30% of genes may be affected by miRNAs; thus miRNAs can potentially regulate thousands of genes.[9] miRNAs act like master regulators of gene expression for many important biological pathways including EMT.[10] miRNAs can act as oncogenes or tumour suppressors depending on their gene targets, and previous studies have shown that miRNA expression is often dysregulated in several cancers including CRC.[10] It has been suggested that the miR-200 family plays a central role in the regulation of the EMT process during cancer progression and metastasis.[1112]

The miR-200 family consists of five members which can be divided into two clusters: miR-200a/b/429 and miR-200c/141, which map to human chromosomes 1 and 12, respectively. The most prominent gene targets of the miR-200 family are *ZEB1* and *ZEB2*, which are direct repressors of the EMT marker, E-cadherin.[11–13] Accumulating evidence suggests that miR-200 family members are involved in cancer progression.[14] Elevated serum levels of miR-200a and miR-200b have been reported in most patients with pancreatic

cancer.[15] In addition, downregulation of miR-141 was observed in gastric cancer tissues compared with matched normal tissue.[16] In spite of the growing evidence highlighting their importance in various cancers, none of the previous studies have systematically investigated the role of the miR-200 family in the development of metastatic disease in human CRC.

In this study, we have addressed this important gap in our knowledge, and have evaluated the functional role of the miR-200 family of miRNAs in a panel of CRC cell lines, as well as through a direct comparison between primary CRCs and corresponding matching liver metastases. Herein, we provide novel evidence that miR-200c acts as a switch that can regulate EMT and MET in human CRC metastasis. In addition, we have performed detailed investigations to uncover the associations between miR-200c and the downstream transcriptional gene targets that mediate distant metastasis in patients with metastatic CRC.

## MATERIALS AND METHODS

### Cell lines and 5-aza-2′-deoxycytidine treatment

Two CRC cell lines with epithelial features (HCT116 and SW480) and two with mesenchymal features (SW620 and RKO) were selected from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) to examine the molecular characteristics of these phenotypes. Cells were treated with 2.5 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma-Aldrich, St. Louis, MO, USA) for 72 h.

### Tissue specimens

A total of 54 formalin-fixed, paraffin-embedded (FFPE) primary CRC tissues and matched corresponding liver metastasis tissues from Okayama University and Toho University in Japan were used in this study (table 1). Written informed consent was obtained from all patients, and the study was approved by the institutional review boards of all participating institutions. Careful microdissection was performed.

### Analysis of miRNA expression

Expression of miR-200b, miR-200c, miR-429 and miR-141 was analysed using TaqMan miRNA assays (Applied Biosystems, Foster City, California, USA). Expression of RNU6B (Applied Biosystems) and miR-16 was used as endogenous controls for cell lines and FFPE tissues, respectively.[17] For in situ hybridisation (ISH), 5 μm-thick FFPE tissue sections were hybridised with the miR-200c probe (LNA-modified and 5'- and 3'-DIG-labelled oligonucleotide; Exiqon, Woburn, Massachusetts, USA), followed by incubation with anti-DIG-AP Fab fragments conjugated to alkaline phosphatase, and the hybridisation signal was detected by applying nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colour substrate (Roche Applied Science, Mannheim, Germany). Positive controls (U6 small nuclear RNA (snRNA), LNA-modified and 5'- and 3'-DIG-labelled oligonucleotide) and negative controls (scrambled miRNA control, LNA-modified and 5'- and 3'-DIG-labelled oligonucleotide) were included in each hybridisation procedure.

### Analysis of DNA methylation

Methylation levels of miRNA promoter regions (miR-200b/429 and miR-200c/141) were analysed by quantitative bisulfite pyrosequencing using the PSQ HS 96A pyrosequencing system (Qiagen, Valencia, CA, USA), as described previously.[18] Primer sequences and PCR conditions are described in online supplementary table 1.

### Analysis of gene expression

Total RNAs were reverse transcribed to cDNA using the Advantage RT PCR Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). For quantitative real-time PCR (qRT-PCR), target gene expression was normalised to glyceraldehyde-3-phosphate dehydrogenase expression. Primers are described in online supplementary table 1.

### Analysis of protein expression

Western blotting was performed using antibodies against E-cadherin (BD Biosciences, Bedford, Massachusetts, USA), vimentin (BD Biosciences), ZEB1 (Santa Cruz, Santa Cruz, CA, USA), ETS1 (Santa Cruz) and FLT1 (Abcam, Cambridge, UK). For immunohistochemical staining, anti-E-cadherin (Dako, Carpinteria, CA, USA) and anti-vimentin (Dako) were exposed according to the manufacturer's recommendations of the iVIEW DAB Detection Kit (Ventana Medical Systems, Tucson, AZ, USA).

### Transfection of miR-200c precursor molecules

In order to induce miR-200c expression, cells were transfected with *hsa-miR-200c* precursor (Applied Biosystems) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To verify the transfection effect of *hsa-miR-200c* precursor, Pre-miR miRNA Precursor Negative Control (Applied Biosystems) containing a random sequence with no identifiable effects on known miRNA function was included in each transfection experiment.

### Proliferation and viability assays

Cell proliferation index was measured by bromodeoxyuridine (Cell Proliferation ELISA, BrdU: Roche) and the MTT assay (Sigma, USA) were performed 3 days after transfection of the miR-200c precursor following the manufacturer's instructions.

### Cell invasion, migration and wound healing assays

At 3 days after transfection with miR-200c precursors or negative controls, invasion and migration assays were performed using Boyden chambers (BD Biosciences) using 8 μm-pore size membranes with Matrigel (for invasion assays) or without Matrigel (for migration assays). For wound-healing assays, cell monolayers transfected with miR-200c precursor were scratched with a clean pipette tip, and cell migration was observed for up to 24 h.

### 3'-UTR reporter luciferase assays

Cells in 12-well plates were co-transfected with luciferase vectors (empty luciferase vector, luciferase vector containing wild-type target gene 3'-UTR, and luciferase vector containing mutant-type target gene 3'-UTR) for ZEB1,[11] ETS1,[19] FLT1[2021] and either miR-200c or negative control precursors using Lipofectamine-2000 (Invitrogen), respectively. pGL3- and pCI-neo-hRL-control vectors were co-transfected as normalisation controls. After 72 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA). Data are presented as ratios between firefly and *Renilla* fluorescence activities.

### Statistical analysis

Data were analysed with GraphPad Prism 5.0 software. To evaluate significant differences between two matched pair groups or between two independent groups of samples, paired t tests and Mann-Whitney U test were used, respectively. Pearson's correlation coefficient (r) was used to measure correlation, and logarithmic regression was used to calculate the $R^2$and to create the equation of the slope.

# RESULTS

## Gain of epithelial characteristics and simultaneous loss of mesenchymal features correlate with miR-200c and miR-141 expression in CRC cell lines

To determine the EMT status in HCT116, RKO, SW480 and SW620 human CRC cell lines, expression of an epithelial marker (E-cadherin) and a mesenchymal marker (vimentin) was determined by qRT-PCR and western immunoblotting (figure 1A). HCT116 and SW480 showed robust expression of E-cadherin, which is consistent with an epithelial phenotype. In contrast, SW620 cells (which were derived from a lymph node from the patient used as the source of SW480 cells) primarily expressed vimentin, which is representative of their mesenchymal character. Owing to methylation-induced transcriptional silencing of the promoter region, RKO cells did not express transcripts for either E-cadherin or vimentin.[22]

To better understand the relationship between the expression patterns of both EMT markers with that of the miR-200 family of miRNAs, we measured the expression of each miR-200 family member by qRT-PCR in all four CRC cell lines (figure 1B). Expression of miR-200b and miR-429, which are part of a cluster on chromosome 1, failed to demonstrate a significant relationship with the expression of either EMT marker in any of the cell lines. On the other hand, expression of the second cluster of miRNAs, mapped to chromosome 12, miR-200c and miR-141, correlated with both EMT markers. The expression pattern of both miRNAs correlated positively with the epithelial marker, E-cadherin, while an inverse relationship was found for the mesenchymal marker, vimentin. These data are of significance, as they suggest that the expression of miR-200c and miR-141 are associated with gain of epithelial features and simultaneous loss of mesenchymal characteristics in CRC cells, the two distinguishing features that are essential for the development of a metastatic phenotype.

## The miR-200c/141 cluster is significantly upregulated in liver metastasis tissues from CRC patients

To confirm our findings in CRC cell lines, we tested our hypothesis for the potential role of the miR-200 family in tissue specimens. As shown in figure 1C, we analysed the expression levels of all four miR-200 family members in 54 pairs of matched primary CRCs (PC) and their corresponding liver metastases (LM). In qRT-PCR analysis, miR-200b was significantly downregulated in LM compared with PC (PC=0.56 vs. LM=0.47; p=0.0320, paired t test). Expression levels of miR-429 did not show any differences between the tissues. On the other hand, miR-200c expression was significantly higher in LM compared with PC (PC=1.31 vs. LM=1.59; p=0.0014, paired t test). Likewise, miR-141 expression was significantly upregulated in liver metastasis compared with primary CRC tissues (PC=0.14 vs. LM=0.17; p=0.0234, paired t test). Interestingly, miR-200c (mean level=1.59) showed higher levels of expression than the other three miR-200 family members (mean values range from 0.01 to 0.47) in the LM.

Next we determined the relationship between the expression levels of individual miRNAs in each of the two miR-200 clusters. The expression of miR-200b and miR-200c correlated positively with that of miR-429 and miR-141, respectively (figure 1D). These data confirm that miR-200b/429 and miR-200c/141 were clustered by expression patterns, and that the miR-200c/141 cluster as a whole is expressed at a higher level than miR-200b/429 in LM.

To further confirm the pathological expression pattern of miR-200c in clinical tissue specimens, ISH staining was performed in primary CRCs with and without metastasis (figure 2B), as well as in matched PC and LM tissues (figure 2C). ISH analysis revealed that early stage CRCs without metastasis and adjacent normal colonic mucosa showed similar expression of miR-200c. On the other hand, late stage CRCs with metastasis revealed low

expression of miR-200c compared with adjacent normal colonic mucosa. These ISH expression data were consistent with our qRT-PCR results for miR-200c expression in primary CRC with metastasis (n=29) and without metastasis (n=36) (figure 2A). In addition, during ISH analysis of matched primary CRC and liver metastasis, although invading or extravasating tumour cells were not easily distinguishable in the clinical specimens, miR-200c expression gradually weakened from lumen to submucosa in primary CRC, indicating that suppression of miR-200c expression might facilitate higher metastatic potential at the invasive front. Likewise, liver metastasised CRC tissues revealed very high expression of miR-200c compared with adjacent hepatocytes, suggesting that high miR-200c expression permits the active cell proliferation required for the consolidation and colonisation of tumour cells at distant metastatic sites.

## Expression of the miR-200 family members is epigenetically regulated in human CRC metastasis

Recent evidence suggests that expression of miR-200 family members can be epigenetically regulated through methylation of their promoter regions.[15,17,23–25] The promoter regions of the miR-200b/429 and miR-200c/141 clusters contain a CpG island and a CpG-rich region, respectively (figure 3A). Therefore we wanted to know whether the expression pattern of each miRNA within the same cluster is regulated in a similar fashion, and, accordingly, we analysed the extent of CpG methylation of both miR-200 family clusters in CRC cell lines and clinical specimens from PC and LM. Based on our expression analysis (figure 1C), we found that, although both miR-200c and miR-141 showed higher expression in LM than in PC, the expression changes were more significant for miR-200c. Therefore we focused our study on the characterisation of miR-200c function in CRC metastasis.

We discovered that HCT116 cells, which showed the lowest expression of miR-200b and miR-429 compared with other CRC cell lines, were highly methylated (60.9%). In contrast, the other three cell lines showed absent or very low levels of promoter methylation (RKO, 0.9%; SW480, 0.9%; SW620, 1.8%), which is consistent with the higher level of expression observed in these three cell lines (figure 3B). For miR-200c/141 promoter region analysis, RKO (78.2%) and SW620 (78.9%) were highly methylated, whereas HCT116 (1.0%) and SW480 (1.0%) displayed almost negligible methylation. As hypothesised, the methylation status of the miR-200 family correlated inversely with the expression patterns in all four CRC cell lines (figures 1B). To further understand the functional significance of promoter hypermethylation of miR-200 family members, we treated CRC cell lines with the demethylating agent, 5-aza-dC. Demethylation of the miR-200b/429 promoter region (62.2% for untreated controls vs 30.3% for 5-aza-dC) resulted in the upregulation of miR-200b and miR-429 in HCT116 cells (figure 3C,D). Similarly, in RKO and SW620 cell lines, demethylation of the miR-200c/141 promoter region (78.0% for RKO control vs 55.0% for 5-aza-dC; 82.6% for SW620 control vs 56.5% for 5-aza-dC) caused upregulation of both miR-200c and miR-141 expression (figure 3C,D).

To further confirm and validate the biological significance of the epigenetic regulation of miR-200 family members in cell lines, we analysed the methylation status in 54 pairs of PC and matched LM. Pyrosequencing analysis revealed that the miR-200b/429 promoter region was significantly hypermethylated in liver metastasis compared with primary CRCs (31.1% for PC vs 36.4% for LM; $p=0.04$; figure 3E). In contrast, the miR-200c/141 promoter region was significantly hypomethylated in LM compared with PC (61.2% for PC vs 46.7% for LM; $p<0.0001$; figure 3F). Finally, it was reassuring to find that the expression patterns of all four miR-200 family members correlated significantly and inversely with the promoter methylation status (figure 3G). These data indicate that the aberrant methylation of the promoter regions may be one of the mechanisms that regulate the expression of miR-200 family members in human CRC metastasis.

### Restoration of miR-200c promotes cell proliferation but inhibits cell invasion and migration in CRC cells

As described above, we found that miR-200c was not only upregulated in LM compared with PC, it was expressed at the highest levels of all miR-200 family members. In view of these findings, we focused on miR-200c for further functional studies. To gain a mechanistic understanding of the potential role of upregulated miR-200c in liver metastasis, two CRC cell lines (RKO and SW620) with the lowest basal expression of miR-200c were transfected with either pre-miR-200c or a negative control. Re-expression of miR-200c after transfection with miRNA precursors was confirmed by qRT-PCR (figure 4A).

In light of previous evidence that transfection with miR-200 promotes cell growth in human cell lines,[26] we first analysed miR-200c-induced cell proliferation by MTT and BrdU assays in transfected cell lines (figure 4B). In both cell proliferation analyses, re-expression of miR-200c in RKO and SW620 cell lines resulted in increased cell proliferation compared with the negative controls.

Next, we investigated the effect of miR-200c re-expression on the invasion and migration abilities of CRC cell lines. Overexpression of miR-200c significantly reduced cell invasion into Matrigel-coated transwell membranes (figure 4C). Similarly, in Boyden chamber assays, the migratory ability of CRC cell lines was suppressed in response to overexpression of miR-200c (figure 4D). Similar results were seen in wound-healing assays, in which miR-200c expression in CRC cell lines markedly reduced the ability to migrate in a scratch assay in monolayer cultured cells (figure 4E). Taken together, these results demonstrate that miR-200c expression induces cell proliferation and at the same time suppresses cell invasion and migration.

### Expression of EMT-related genes is negatively regulated by miR-200c in CRC cell lines

EMT occurs during tumour progression, which provides cancer cells with invasive and metastatic properties. Several putative targets of the miR-200 family have been suggested in other cancers, but none of the previous studies has investigated miRNA–mRNA associations in CRC.[111219–21] Three EMT-related genes (zinc finger E-box-binding homeobox 1 (*ZEB1*, figure 5A), v-ets erythroblastosis virus E26 oncogene homologue 1 (*ETS1*, figure 5B) and fms-related tyrosine kinase 1 (*FLT1*, figure 5C)) have been proposed as putative targets of miR-200c on the basis of in silico miRNA target prediction programs (TargetScan, PicTar, miRanda and miTarget). However, as none of these targets has been functionally validated, we determined the relationship between the expression of these three EMT-related genes and miR-200c expression in CRC cell lines. We found that the overexpression of all three putative target genes (*ZEB1, ETS1* and *FLT1*) was markedly reduced by miR-200c expression in RKO and SW620 transfected with pre-miR-200c (figure 5A–C).

To clarify the interaction between miR-200c and the functional miR-200c seed regions within the 3 □UTR region of the target genes (*ZEB1, ETS1* and *FLT1*), we performed luciferase reporter assays. The luciferase activity of wild-type reporter plasmids was significantly decreased by the miR-200c precursor, whereas the luciferase activity of mutant-type reporter plasmids remained unchanged by miR-200c precursors (figure 5A–C).

To further examine whether miR-200c overexpression-induced inhibition of EMT-related genes actually inhibits the EMT phenotype, we confirmed the expression of E-cadherin and vimentin (figure 5D) in miR-200c-transfected SW620 cells; RKO cells do not express either gene because of methylation-induced silencing. Overexpression of miR-200c significantly increased E-cadherin expression, with a simultaneous decrease in the expression of vimentin in SW620 cells. Our findings suggest that miR-200c overexpression leads to direct targeting of several EMT-related genes, which in turn regulate the metastatic behaviour of CRC cells.

### Overexpression of miR-200c induces MET in liver metastasis

Next, we asked whether there was a correlation between *ZEB1, ETS1* and *FLT1* expression in PC and LM. To this end, expression levels of the three miR-200c target genes were measured by qRT-PCR in PC and corresponding LM (figure 6A–C). The expression of the *ZEB1, ETS1* and *FLT1* genes was significantly reduced in LM compared with their corresponding PC. Furthermore, the expression levels of all three genes showed an inverse correlation with miR-200c expression in metastatic CRC in the liver.

Finally, we analysed the expression status of E-cadherin and vimentin by qRT-PCR and ISH in matched PC and LM (figure 6D–F). Expression levels of E-cadherin were significantly increased in LM compared with the PC (p=0.002). In contrast, expression of vimentin was significantly decreased in LM (p=0.024). These findings have important biological and clinical implications, as they suggest that upregulation of miR-200c in CRC metastases to the liver results in the MET phenotype, during which the metastatic colonic cells increasingly gain epithelial features (increased E-cadherin expression) and simultaneously lose mesenchymal characteristics (reduced vimentin expression), which facilitates their proliferation and expansion subsequent to settlement at the distant metastatic site.

## DISCUSSION

This study describes the role of the miR-200 family in the progression of CRC from a primary process to metastatic disease, and highlights the importance of upregulation of the miR-200c/141 cluster in liver metastases from CRC. This is the first study to directly analyse the miR-200 family of miRNAs in primary cancer tissues (CRC in this instance) and matching metastatic tissues (liver) and shows a sequence in which this group of miRNAs is initially silenced, permitting behaviours that facilitate escape from the primary tumour, and then reverse the phenotype, which facilitates the growth of metastatic lesions. We demonstrate that miR-200c/141 expression correlates with increased E-cadherin and reduced vimentin expression, suggesting that during cancer metastasis, CRC cells initially lose epithelial features while simultaneously gaining the mesenchymal characteristics required for the EMT. Our data on the functional characterisation of miR-200c gene targets lend further credence to our hypothesis, which suggest that increased expression of miR-200c results in the negative regulation of its gene targets (*ZEB1, ETS1* and *FLT1*), which in turn regulate E-cadherin and vimentin expression to trigger an EMT switch in CRC cells. We provide further insight into the molecular underpinnings of the metastatic process and illustrate that subsequent to the establishment of an EMT phenotype, which permits the migration of CRC cells to the liver, the reverse process of MET subsequently ensues. These data highlight the significance of EMT–MET switching, as MET is an essential component of metastatic disease, and enables metastasised clones of cells to regain epithelial features, which permits their proliferation and expansion in the newly formed metastatic foci.

Previous work from our group and others has suggested that aberrant DNA methylation is one of the important mechanisms that regulate the expression of several human miRNAs, including miR-200, in various cancers.[151723–252728] However, the epigenetic mechanisms involved in the regulation of the miR-200 family in CRC remain unclear. In this study, we address this gap in knowledge, and demonstrate that both miR-200b/429 and miR-200c/141 clusters are epigenetically regulated in primary CRCs. With regard to epigenetic regulation of miR-200 family members in CRC metastasised to liver, we made a very unique observation. Among the miR-200 family of miRNAs, miR-200c appears to play the most critical role in mediating distant metastasis to liver in patients with CRC. This conclusion draws support from the following observations. First, among all miR-200 family members analysed, miR-200c was expressed at the highest levels in tissue specimens from CRC patients. Second, miR-200c was significantly downregulated in primary CRC with

metastasis compared with CRC without metastasis, which was consistent with markedly low in situ expression of miR-200c at the invasive front of late-stage primary CRCs. Third, miR-200c was significantly upregulated in metastatic CRC in the liver, suggesting its importance in this process, which correlated with hypomethylation of its promoter region. Collectively, these data highlight the importance of miR-200c overexpression and its epigenetic regulation in metastatic CRC, which may be an important initial step that triggers the MET phenotype which ensues after EMT-mediated emigration of these neoplastic clones of CRC cells to the liver. In addition, transactivation of the miR-200 family by p53[29] and a feedback loop mechanism between miR-200 family members and ZEB1 have also been proposed.[133031] However, the methylation status of the miR-200c promoter region did not correlate with the p53 genotype in CRC cell lines analysed in our study (online supplementary figure 1).

Our observations of hypomethylation-induced overexpression of miR-200c in metastatic CRC are intriguing, but not surprising. Although there has been no direct evidence indicating the role of miR-200 family members in CRC metastasis, morphological analysis of primary CRC and liver metastases revealed that, in each instance, cells with mesenchymal characteristics are often present at the invasive margin, while cells in the centre tend to have a more epithelial morphology.[32] In addition, several studies have demonstrated the effect of miR-200c overexpression in the induction of growth and simultaneous inhibition of invasion and migration in cancer cells.[1112212633–36] These data are of significance as they indirectly support our observation of the appearance of an EMT phenotype in the colon (as centrally located epithelial cells gain mesenchymal features and move to the peripheral invasive margin of the tumour), which later switches to a MET phenotype once the metastatic cells have reached the liver, where mesenchymal cells at the invasive margin begin to regain their epithelial features in the centre of the metastatic locus. We have further discovered and functionally validated novel miR-200c gene targets: *ZEB1, ETS1* and *FLT1*. These genes have been shown to be involved in the regulation of cellular invasiveness and mobility,[213738] and our data demonstrate a reciprocal relationship with miR-200c expression. Taken together, these data indicate that liver metastasis in patients with CRC is mediated by miR-200c via regulation of its gene targets, which initially promotes EMT in the colon, and later initiates the MET process once the cells have reached suitable distant metastatic sites.

On the basis of the findings from this study, we propose a consolidated model that illustrates a plausible sequence of events that orchestrate CRC metastasis to the liver. This model highlights the potential role of miR-200c in regulating the symmetrical molecular events during sequential EMT and MET processes (figure 7). In this model, hypermethylation of miR-200c in primary CRC tissues is a seminal event that triggers the EMT process. During EMT, methylation-induced downregulation of miR-200c allows upregulation of several of its direct target genes, *ZEB1, ETS1* and *FLT1*, in CRC cells. Since these genes are EMT drivers, they impart a gain of invasive and metastatic potential, involving the simultaneous loss of E-cadherin and enhanced vimentin expression in these new metastatic clones. Loss of E-cadherin expression diminishes cell–cell adhesion, permitting newly mobile CRC cells to move freely, enter the blood circulation, and eventually settle at distant metastatic sites, including the liver. To consolidate the settlement of metastasised cells in secondary sites, hypomethylation of miR-200c mediates the MET process. During MET, hypomethylation-induced re-expression of miR-200c suppresses the EMT-driving genes, which is accompanied by high E-cadherin and low vimentin expression. Such a switch from a mesenchymal to an epithelial phenotype permits the metastasised cells to regain their proliferative potential and resume growth at the secondary metastatic sites. EMT and MET are two opposite behaviours that are required for `successful' metastasis. It is a reasonable speculation therefore that such a process might be driven by a reversible genetic alteration

such as promoter methylation. If EMT were driven by a mutation in the DNA sequence, it is difficult to speculate how such a process might be reversible.

In conclusion, this study provides important clues underpinning the mechanism of metastasis in CRC. Our discovery of the pivotal role that miR-200c plays in the metastatic behaviour of CRC cells predicts potential diagnostic and prognostic value for this miRNA as a biomarker. These results may also have implications for the clinical management of patients with metastatic disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Significance of this study

**What is already known on this subject?**

- Distant metastasis is a major cause of cancer-related mortality in patients with colorectal cancer (CRC).
- Epithelial-to-mesenchymal transition (EMT) is an essential step in mediating distant metastasis in human cancers.
- Recent evidence suggests that the miR-200 family of microRNAs is involved in cancer progression; however, no previous study has investigated the role of miR-200 members in the development of metastatic disease in human CRC.

**What are the new findings?**

- This study describes the role of the miR-200 family in the progression of CRC from a primary lesion to metastatic disease, and suggests the importance of hypomethylation and upregulation of the miR200c/141 cluster in liver metastases from CRC.
- Low in situ miR-200c expression at the invasive front of primary CRCs and high miR-200c expression in liver metastases highlight a crucial role for miR-200c in CRC metastasis.
- Functional characterisation of miR-200c gene targets revealed that overexpression of miR-200c results in the negative regulation of its gene targets (*ZEB1, ETS1* and *FLT1*), which in turn upregulates E-cadherin and downregulates vimentin expression to facilitate gain of epithelial characteristics that are required for mesenchymal-to-epithelial transition (MET) behaviour in CRC cells.
- This study illustrates that subsequent to the establishment of an EMT phenotype, the reverse process of MET ensues, which enables metastasised clones of cells in the liver to regain their epithelial features, permitting proliferation and expansion in the newly formed metastatic site(s).

**How might it impact on clinical practice in the foreseeable future?**

- Our study highlights a pivotal role for miR-200c in the metastatic behaviour of CRC cells, and suggests that miR-200c may serve as a potential diagnostic biomarker and possible therapeutic target for patients with CRC.

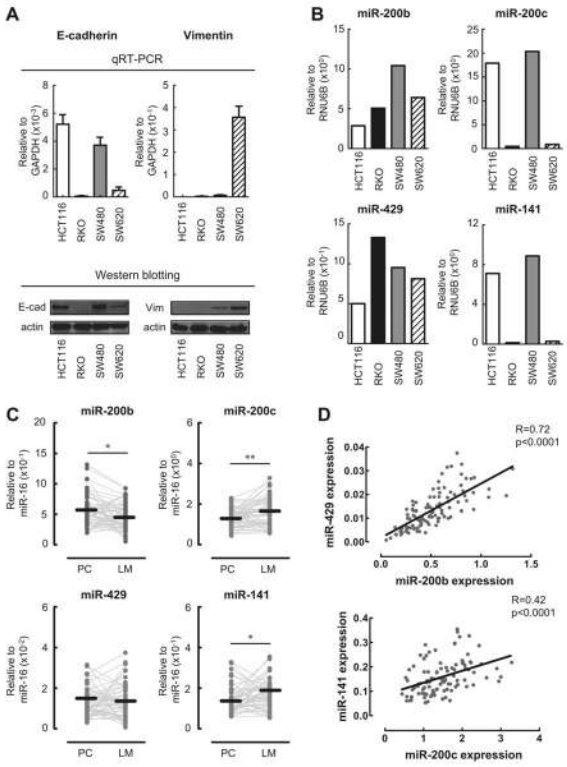


**Figure 1.**

Expression status of miR-200 family members in colorectal cancer (CRC) cell lines and human tissue specimens. (A) Expression of mesenchymal (vimentin (Vim)) and epithelial (E-cadherin (E-cad)) markers in CRC cell lines (HCT116, RKO, SW480 and SW620). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT, quantitative real time. (B) Expression status of miR-200 family members (miR-220b, -429, -200c and -141) in CRC cell lines. (C) Expression of each miR-200 family member in matched primary CRC (PC) and their corresponding liver metastasis (LM). The bold horizontal bar represents mean expression levels; *p<0.05, **p<0.01, t test. (D) Each of the four miR-200 family members were clustered together on the basis of genomic location, and expression levels were compared. Pearson's correlations were calculated for each miRNA cluster: miR-429/200b (upper panel) and miR-200c/141 (lower panel).

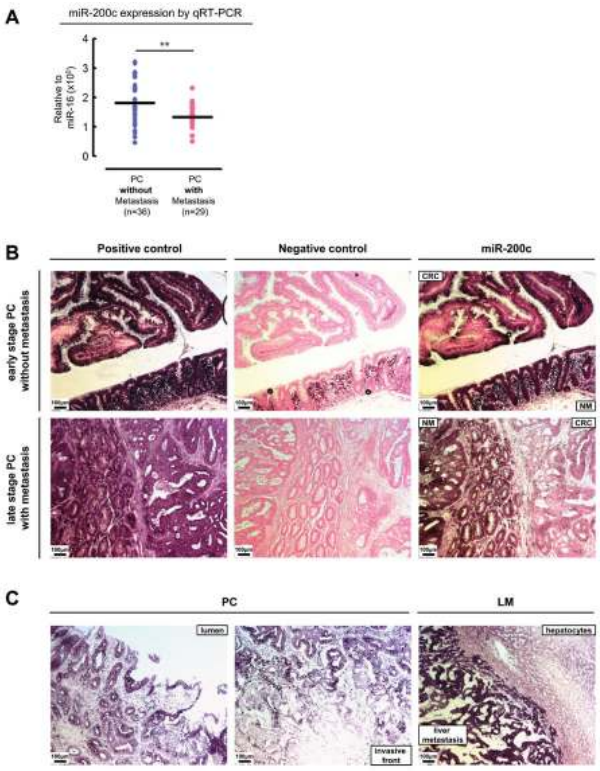


**Figure 2.**

The expression of miR-200c in primary colorectal cancer and liver metastasis tissue specimens. (A) Quantitative expression analysis of miR-200c by quantitative real-time (qRT) PCR in primary CRC (PC) with and without metastasis (**p<0.01). (B) In situ hybridisation analysis of miR-200c in PC with and without metastasis. Normal mucosa, NM; positive control, U6 snRNA; negative control, scrambled miRNA control. (C) In situ hybridisation analysis of miR-200c in matched PC and their corresponding liver metastasis (LM).

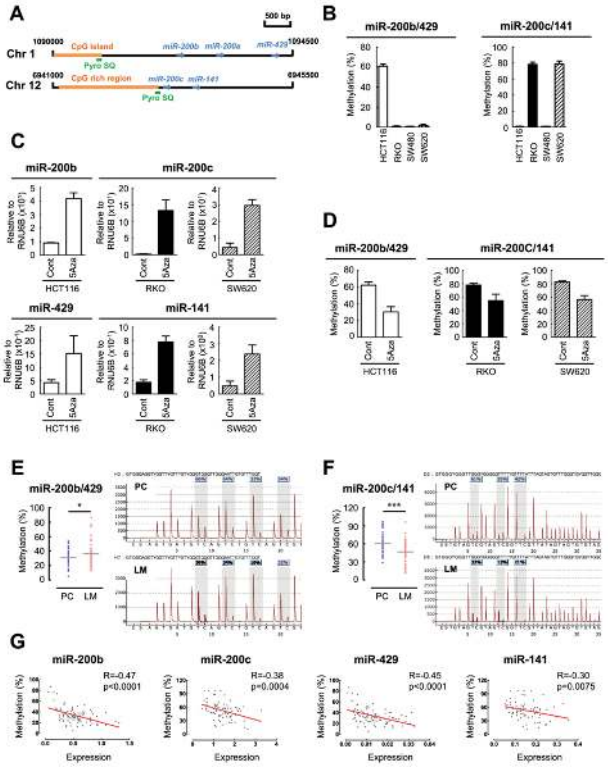


**Figure 3.**

Methylation analysis of miR-200b/429 and miR-200c/141 clusters. (A) Schematic illustration of miR-200b, -200c, -141 and -429 genomic loci. Blue arrows represent miRNA mapping locations; orange bars indicate a CpG island or CpG-rich regions; green bars represent pyrosequencing primer location. (B) Methylation status of miR-200b/429 and miR-200c/141 promoter CpG regions in colorectal cancer (CRC) cell lines. (C) TaqMan real-time PCR expression analysis in CRC cell lines treated with 5-aza-2 □deoxycytidine (5-aza-dC; 5Aza). (D) Quantitative methylation pyrosequencing analysis in CRC cell lines treated with 5-aza-dC. (E) Methylation analysis of the miR-200b/429 promoter region in 54 paired primary CRC (PC) and their corresponding liver metastasis (LM). Grey horizontal bars represent the mean methylation levels; grey vertical boxes in the pyrograms illustrate individual CpG sites analysed; *p<0.05, t test. (F) Methylation analysis of the miR-200c/141 promoter region in 54 paired PC and LM. ***p<0.001, t test. (G) Correlation analysis between methylation levels (%) and expression status of individual miR-200 family members. Red line represents linear regression line.

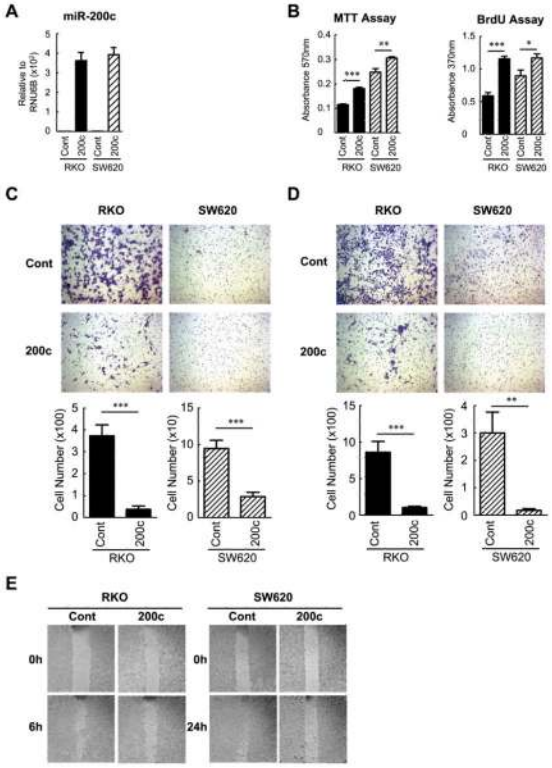


**Figure 4.**

Functional analysis of miR-200c. (A) Expression of miR-200c following transfection with pre-miR-200c was confirmed by TaqMan real-time PCR. (B–E) Data from various assays following transfection with pre-miR-200c in colorectal cancer (CRC) cell lines. (B) MTT and bromodeoxyuridine (BrdU) cell proliferation assays (C) Cell invasion assays using Matrigel-coated transwell membranes (upper panel, representative pictures of invasion chambers; bottom panel, average counts from four random microscopic fields). (D) Cell migration assays using transwell (upper panel, representative pictures of migration chambers; bottom panel, average counts from four random microscopic fields). (E) Wound-healing assay. Cell monolayers were scratched with a pipette tip and images were taken 0, 6 and 24 h after wound formation. *p<0.05, **p<0.01, ***p<0.001, t test.

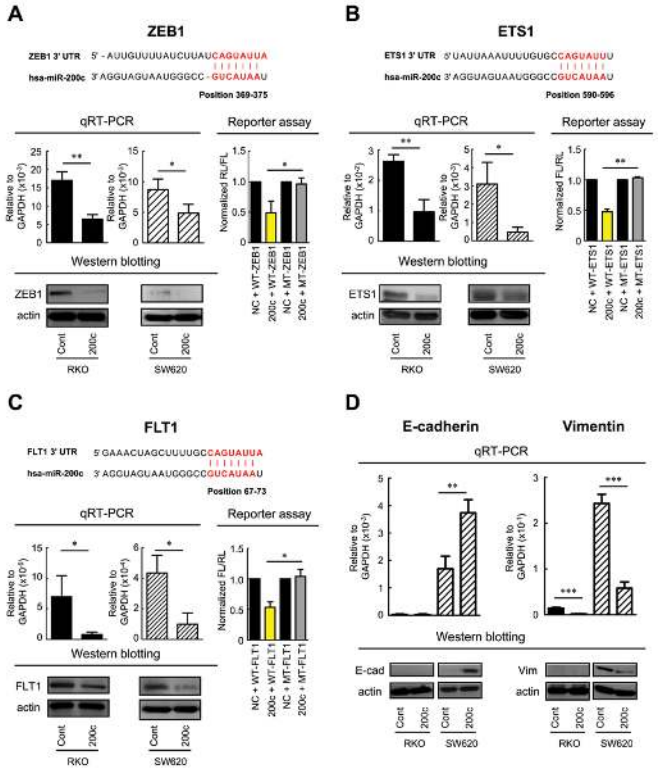


**Figure 5.**

Predicted miR-200c target genes and validation in colorectal cancer (CRC) cell lines. (A–C) Overexpression of miR-200c-suppressed mRNA (quantitative real-time (qRT)-PCR) and protein expression (western blotting) of *ZEB1, ETS1* and *FLT1*, respectively. Luciferase reporter assays illustrate direct binding of miR-200c to the wild-type (WT), but not the mutant (MT) sequences within the 3 UTR regions of *ZEB1, ETS1* and *FLT1*, respectively. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Cont, Control; 200c, transfection of pre-miR-200c; NC, negative control; FL, firefly luciferase; RL, *Renilla* luciferase. (D) Overexpression of miR-200c increased the expression of the epithelial marker (E-cadherin) but decreased the expression of the mesenchymal marker (vimentin) in CRC cell lines. *p<0.05, **p<0.01, ***p<0.001, t test.

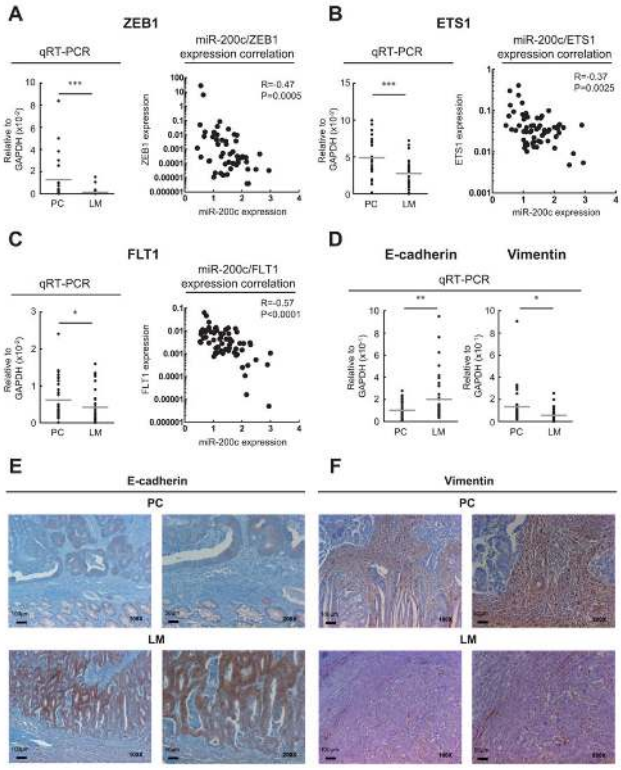


**Figure 6.**

Expression status of epithelial-to-mesenchymal transition (EMT)-related genes in tissue specimens. (A–C) mRNA (quantitative real-time PCR (qRT-PCR)) expression status of EMT-related genes (*ZEB1, ETS1* and *FLT1*) in primary colorectal cancer (CRC) (PC) and matched liver metastasis (LM); miR-200c/target gene (*ZEB1, ETS1* and *FLT1*) expression correlation in CRC tissues. (D) mRNA expression status of E-cadherin and vimentin in PC and LM. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (E) Protein expression status of E-cadherin in PC and LM. (F) Protein expression status of vimentin in PC and LM. Grey horizontal bars in each graph represent the mean expression levels; *p<0.05, **p<0.01, ***p<0.001, t test.

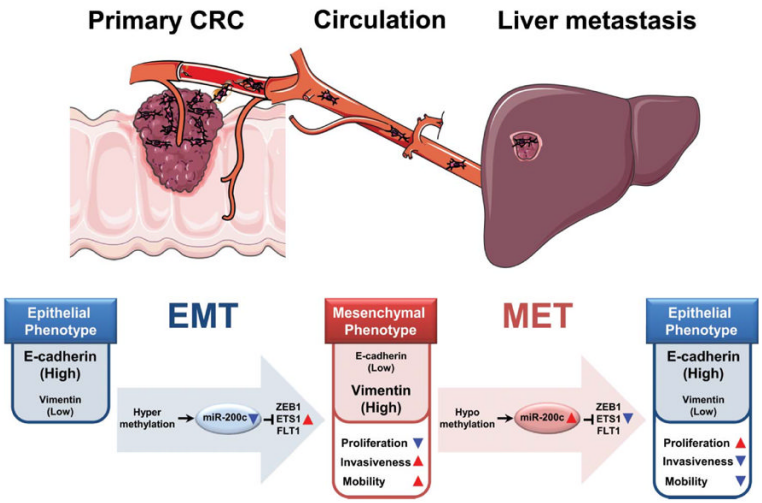


**Figure 7.**

A consolidated model for the role of miR-200c in colorectal cancer (CRC) metastasis. In this model, hypermethylation of miR-200c in the CRC triggers an epithelial-to-mesenchymal transition (EMT) phenotype. During EMT, the hypermethylation of miR-200c allows upregulation of several direct target genes (*ZEB1, ETS1* and *FLT1*). This in turn allows cells to acquire mesenchymal characteristics, which involves the simultaneous loss of E-cadherin and enhanced expression of vimentin. Alterations in E-cadherin expression allows cells to enter the blood circulation and eventually settle at distant metastatic sites, including the liver. Metastasised liver cells become hypomethylated at the miR-200c locus, which initiates the mesenchymal-to-epithelial transition (MET) process. Subsequently, re-expression of miR-200c causes suppression of EMT-driving genes, which accompanies high E-cadherin and low vimentin expression.

## Table 1

Clinicopathological characteristics of patients with colorectal cancer

| Characteristic | No of patients (N=54) |
|---|---|
| Sex | |
| Male | 31 |
| Female | 23 |
| Age (years) | |
| ≤65 | 34 |
| >65 | 20 |
| Histological type | |
| Well differentiated | 14 |
| Moderately differentiated | 39 |
| Mucinous | 1 |
| Tumour location* | |
| Proximal | 10 |
| Distal | 18 |
| Rectum | 26 |
| Stage | |
| I + II | 7 |
| III + IV | 47 |
| Microsatellite instability | |
| MSS | 50 |
| MSI | 4 |

MSI, microsatellite instable tumour; MSS, microsatellite stable tumour.

*In relation to the splenic flexure.