MicroRNA-145 inhibits tumour growth and metastasis in colorectal cancer by targeting fascin-1

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Background: Recent studies have reported miR-145 dysregulated in colorectal cancer (CRC). In this study, miR-145 profiles were compared between CRC and corresponding non-tumour tissues.

Methods: The expression levels of miR-145 were analysed in CRC cell lines and tumour tissues by real-time PCR. A luciferase reporter assay confirmed direct targets. The functional effects of miR-145 were examined in transfected CRC cells in vitro and in vivo using established assays.

Results: Downregulation of miR-145 was detected in most primary CRC tumours, and was significantly correlated with a more aggressive phenotype of CRC in patients. In CRC cell lines, ectopic overexpression of miR-145 inhibited cell proliferation, motility and invasion in vitro. Stable overexpression of miR-145 suppressed tumour growth and pulmonary metastasis in vivo. Further studies indicated that miR-145 may directly interact with the 3’-untranslated region (3’-UTR) of Fascin-1 messenger RNA (mRNA), downregulating its mRNA and protein expression levels. In clinical specimens, Fascin-1 expression was negatively correlated with miR-145 expression.

Conclusions: MiR-145 has a critical role in the inhibition of invasive and metastatic capacities of CRC, probably through directly targeting Fascin-1. This miRNA may be involved in the development and progression of CRC.

Colorectal cancer (CRC) is one of the most common malignancies worldwide (Weitz et al., 2005), with a high capacity for tumour migration and invasion. The development of CRC from normal epithelial cells to malignant carcinomas is believed to be a multistage process involving genetic changes, leading to the activation of oncogenes and inactivation of tumour suppressor genes. Although migration and invasion have been acknowledged as the most lethal attributes of solid tumours, the underlying molecular mechanisms are largely unknown. Therefore, the identification of novel molecules that are differentially expressed in CRC cells may provide insights into the mechanisms involved.

Recently, the categories of cancer-related genes have been expanded to include microRNAs (miRNAs) (Esquela-Kerscher and Slack, 2006; Medina and Slack, 2008; Visone and Croce, 2009). This large family of highly conserved small non-coding RNAs may regulate a vast number of protein-coding genes, including...
oncogenes and tumour suppressor genes, which suggests that miRNAs can function as tumour suppressors or oncogenes. Many miRNAs are highly tissue-specific and important for cell development and differentiation. As such, the aberrant expression of miRNAs can lead to cellular dedifferentiation, oncogenesis, cancer metastasis and tumour invasion (Esquela-Kerscher and Slack, 2006). The first report of the involvement of an miRNA in CRC appeared in 2003, when Michael et al (2003) found the reduced accumulation of miR-145 in colorectal neoplasia compared with normal mucosa. Since then, there have been frequent reports of aberrant miRNA expression in CRC (Akao et al, 2007; Lanza et al, 2007; Asangani et al, 2008; Yang et al, 2009), suggesting a close correlation between miRNAs and the development, progression, metastasis, and prognosis of CRC. MiR-145 has been found to be downregulated in CRC, and has therefore been proposed as a metastasis-suppressor miRNA (Luo et al, 2011). MiR-145 has mostly been reported as downregulated among the 164 miRNAs that have been identified as dysregulated, and has been reported to regulate a set of metastatic genes that includes ADAM17 (Lu et al, 2013), Fascin-1 (Liu et al, 2012), and mucin 1 (MUC1) (Sachdeva and Mo, 2010). Despite an increasing number of studies on the biogenesis and mechanisms of miR-145 in the pathogenesis of CRC, the mechanisms of miR-145 dysregulation are still unclear.

### MATERIALS AND METHODS

#### Cell culture and transfection

Five human CRC cell lines (SW480, HT29, LS174T, SW620, and LoVo) and the human embryonic kidney cell line, 293T, were acquired from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS; Hy-Clone, Logan, UT, USA). Two normal colon epithelial cell lines (FHC and NCM460) were grown in DMEM:F12 (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS, or in M3:10TM medium (INCELL, San Antonio, TX, USA) supplemented with 10% FBS, respectively.

The miRNAs and siRNAs were purchased from GenePharm (Shanghai, China) and transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

#### Human colorectal tumour tissue specimens

Forty-three of fresh CRC tissue samples (14 colon and 29 rectum) from CRC patients, Human colorectal tumour tissue specimens. (Shanghai, China) and transfected into the cells with Lipofectamine 2000 (INCELL, San Antonio, TX, USA) supplemented with 30% fetal bovine serum (FBS; Hy-Clone, Logan, UT, USA). Two normal colon epithelial cell lines (FHC and NCM460) were grown in DMEM:F12 (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS, or in M3:10TM medium (INCELL, San Antonio, TX, USA) supplemented with 10% FBS, respectively.

The miRNAs and siRNAs were purchased from GenePharm (Shanghai, China) and transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

#### Cell proliferation analysis

Cells (1 × 10^4) were seeded in 96-well plates in triplicate, with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) working solution, and incubated for 4 h at 37°C. The medium was then removed and 100 μl of dimethyl sulphoxide was added to dissolve the formazan crystals. Cell viability was assessed for five consecutive days by absorbance at 570 nm using a microplate reader (model 680 Microplate Reader, Bio-Rad, Gaithersburg, MD, USA). All experiments were repeated three times and the averages were calculated.

#### Luciferase assays

Fourteen vectors, including a full-length 3'-untranslated region (3'-UTR) of Fascin-1 (FSCN1) mRNA (position 51–1180) and four mutated full-length 3'-UTR of Fascin-1 mRNA in which putative miR-145-binding sites were deleted, were transfected into cells with either miR-145 or negative control miRNA. After culturing for 48 h, the cells were collected, washed with PBS, and lysed with luciferase lysis buffer. The luciferase activities were normalized to the renilla luciferase activity. The firefly luciferase activities were measured using a Dual-Glo Luciferase Assay System (Promega). The results were expressed as the relative luciferase activity (firefly luciferase activity/renilla luciferase activity) for each construct compared with that of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The results were expressed as the relative luciferase activity (firefly luciferase activity/renilla luciferase activity) for each construct compared with that of the pmirGLO Vector.
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Immunoblotting. Cells were harvested 72 h post transfection and lysed in RIPA buffer. Cell lysates were separated by electrophoresis on 12% SDS–polyacrylamide gels, transferred to PVDF membranes (Millipore, Billerica, MA, USA), and probed with primary antibodies: mouse to Fascin-1 (1:1000) (Abcam, Cambridge, MA, USA) and mouse to anti-β-actin (1:2000) (Cell Signaling Technology, Danvers, MA, USA). After washing, the membranes were incubated with secondary antibody HRP-conjugated goat anti-mouse (1:5000 dilution; Invitrogen) and visualised by enhanced chemiluminescence.

Immunohistochemistry and immunohistochemical staining. Detection of Fascin-1 was performed on 5-μm FFPE CRC tissue sections. Antigen retrieval was performed in Tris-EDTA buffer using the microwave protocol. Tissues were incubated with primary antibody mouse to Fascin-1 (1:250) (Abcam) at room temperature for 30 min, then stained using the EnVision Detection Kit, peroxidase/DAB, Rabbit/Mouse (Gene Tech, Shanghai, China). Immunohistochemical scores were allocated according to a semi-quantitative scale based on previous criteria (Hashimoto et al., 2006). In each case, 10 high-power fields of representative areas were counted. The staining was evaluated independently by two pathologists and any discrepancies were resolved by consensus.

Lentivirus packaging and transduction. MiR-145 and control miRNA precursor sequences were amplified from human genomic DNA and cloned into the lentiviral vector pLVX-shRNA1 (Clontech Laboratories, Inc., Palo Alto, CA, USA). Virus packaging was performed in 293T cells. pLV-miR-145 or pLV-miR-control and Lenti-X HTX Packaging Kit (Clontech Laboratories, Inc.) were co-transfected using the Xfect transfection reagent according to the manufacturer’s protocols. The SW620H cells were transduced with pLV-miR-145 or pLV-miR-control. Forty-eight hours after infection, 2 μg ml⁻¹ of puromycin was added to the media for 2 weeks to select the cells infected with the lentivirus. The cell line that stably expressed miRNA-145 was named LV-miR145-SW620H, and the control vector cell line was named LV-miRcontrol-SW620H. Real-time PCR assays were used to detect the expression of miR-145 in the two stable cell lines as described above.

Proliferation and metastasis assays in vivo. Female athymic BABL/c nude mice (4–6 weeks old) were used under conditions approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. To determine the proliferation capacity of LV-miR145-SW620H and LV-miR-control-SW620H in vivo, a total of 1×10⁶ cells were injected subcutaneously into nude mice (n=8). Tumour volume (V) was calculated as follows: V = length × width² × 0.5. To investigate experimental lung metastasis, tumour cell suspensions (1.5×10⁶ cells per mouse) were injected into the lateral tail veins of each anaesthetised nude mouse (n=8). Six weeks after injection, the animals were killed; lungs were dissected and paraffin embedded, and 5-μm sections were stained with haematoxylin and eosin. The metastases were counted in a double-blind manner with the aid of a dissecting microscope as described previously (Huang et al., 1998).

Statistical analysis. All data were analysed using Prism 5.0 software (GraphPad, La Jolla, CA, USA) and presented as mean ± s.e. The significances of the observed differences were determined by the Student’s t-test or by one-way analysis of variance. The relationship between miR-145 and Fascin-1 mRNA or protein was analysed by correlation coefficients and linear regression analysis. P<0.05 was considered statistically significant.

RESULTS

MiR-145 is specifically downregulated in human metastatic CRC cell lines and colorectal tumours. SYBR green qPCR was performed to detect miR-145 levels in CRC cell lines and tissues. Expression levels of miR-145 were tested in seven human colonic cell lines: normal colon epithelial cell lines (FHC, NCM460); tumorigenic but non-metastatic CRC cell lines (HT29, LS174T, SW480); and
MiR-145 expression suppresses metastasis-relevant traits in vitro. Given the inverse correlation between miR-145 levels and malignant phenotype, the anti-metastatic roles of miR-145 in CRC were tested. SW620 and LoVo cells transfected with miR-145 mimics exhibited high levels of miR-145 compared with normal colon epithelial cells (FHC) (Figure 2A). The results showed that ectopic miR-145 significantly reduced cell proliferation (Figure 2B), migration, and invasion (Figure 2C), but did not affect cell cycle distribution in vitro (Figure 2D).

Table 2. Clinicopathological parameters in clinical CRC cases

| Variable                  | Cases (n) | %   | Expression level of miR-145 | P-value  |
|---------------------------|-----------|-----|----------------------------|----------|
| Age (years)               |           |     |                            |          |
| < 60                      | 19        | 44.19 | –10.54 ± 1.96              | 0.614    |
| ≥ 60                      | 24        | 55.81 | –10.22 ± 2.14              |          |
| Sex                       |           |     |                            |          |
| Male                      | 27        | 62.79 | –10.16 ± 2.35              | 0.418    |
| Female                    | 16        | 37.21 | –10.69 ± 1.42              |          |
| Family history of CRC     |           |     |                            |          |
| Yes                       | 6         | 13.95 | –10.02 ± 1.94              | 0.667    |
| No                        | 37        | 86.05 | –10.41 ± 2.09              |          |
| Tumour location           |           |     |                            |          |
| Colon                     | 14        | 32.56 | –10.05 ± 2.02              | 0.497    |
| Rectum                    | 29        | 67.44 | –10.51 ± 2.01              |          |
| Lymph node metastasis     |           |     |                            |          |
| Yes                       | 26        | 60.47 | –11.29 ± 1.54              | 0.000    |
| No                        | 17        | 39.53 | –8.96 ± 1.91               |          |

Abbreviation: CRC – colorectal cancer.
MiR-145 inhibits growth and metastasis in CRC

Inhibition of miR-145 promotes metastasis-relevant traits in vitro. It was then determined whether miR-145 prevented metastatic-relevant traits from being acquired by non-metastatic human CRC cells. MiR-145 was transiently inhibited in non-metastatic SW480 and HT-29 cells with antisense oligonucleotides (Figure 3A). The suppression of miR-145 enhanced cell migration, invasion (Figure 3B), and cell proliferation (Figure 3C), but not cell cycle distribution (Figure 3D).

MiR-145 directly regulates Fascin-1 in CRC cells. MiR-145 may impair the metastatic capacity of CRC cells by regulating metastasis-related genes; therefore, miRNA-PicTar and TargetScan algorithms were used to predict the functional target genes of miR-145. Four metastasis-related genes were selected based on their 3′-UTR to test using the luciferase assay: ADAM17; NEDD9; Fascin-1; and mucin 1 (MUC1). MiR-145 only significantly repressed the luciferase activity of full-length 3′-UTR of Fascin-1 mRNA in SW620 cells (Figure 4A). To determine the specific sites targeted by miR-145, the four putative miR-145-binding sites at the 3′-UTR of Fascin-1 were cloned downstream of a firefly luciferase cassette. The luminescence intensity was significantly decreased in miR-145 transfectants with two putative miR-145 sites (positions 116–122 and 377–383, respectively); however, by mutating the two miR-145-binding sites, the repressive effect of miR-145 was abolished (Figure 4B). In addition, we constructed four mutated vectors in which the putative sites targeted by the miR-145 were deleted, and the results of the luminescence intensity were in accordance with the above results (Figure 4C). Furthermore, the ectopic expression of miR-145 resulted in significantly reduced Fascin-1 mRNA and protein levels in SW620 and LoVo cells, whereas the inhibition of miR-145 in SW480 and HT-29 cells significantly raised Fascin-1 mRNA and protein levels in both cell lines (Figure 4D and E).

To determine whether miR-145 reduced the migration and invasion capacity of CRC in a Fascin-1-dependent manner, the effect of siRNA targeting of Fascin-1 was examined. The results showed that suppression of Fascin-1 expression by siRNA reduced Fascin-1 protein levels, cell proliferation, invasion, and migration (Figure 5A–C), which is consistent with the inhibitory effects induced by downregulation of miR-145. Furthermore, when SW620 cells were treated with Fascin-1 siRNA in combination with miR-145 mimics (Figure 5A–C), synergistic inhibitory effects were observed compared with treatments with either Fascin-1 siRNA or miR-145 mimics alone. By using an expression construct that encoded the entire Fascin-1-coding sequence, but lacked the 3′-UTR, mRNA that was resistant to miRNA-mediated suppression was produced.

These results showed that the overexpression of Fascin-1 protein with pcDNA-Fascin-1 greatly enhanced the migration and invasion of SW620 and LoVo cells (Figure 5D), and, as predicted, the co-transfection of pcDNA-Fascin-1 and miR-145 mimics into SW620 and LoVo cells significantly rescued miR-145-suppressed migration and invasion.

Inverse correlation of expression between miR-145 and Fascin-1. To confirm the relationship between miR-145 and Fascin-1 expression, miR-145 and Fascin-1 mRNA expression and protein levels were investigated in the seven CRC cell lines and 43 primary CRC and paired adjacent normal tissues. As predicted, the mRNA levels of Fascin-1 were negatively correlated with miR-145 levels (Figure 6A). Correlation analysis of the Fascin-1 protein score with miR-145 expression suggested an inverse relationship (Figure 6B);
however, miR-145 dysregulation could not account for the high expression level of Fascin-1 in some CRC cells.

**MiR-145 inhibits CRC growth and metastasis in vivo.** To further investigate the contribution of miR-145 in vivo, a lentivirus vector was constructed to mediate the expression of miR-145, and two stable cell lines were established named LV-miR145-SW620 and LV-miRcontrol-SW620. These cell lines were injected subcutaneously into the flanks of nude mice, and tumour progression was observed over time. After 6 weeks, the mice in both groups were moribund as a result of primary tumour burdens. The volumes of the tumours resulting from LV-miR145-SW620 injection were significantly smaller than those resulting from LV-miRcontrol-SW620 injection (Figure 7A). In addition, immunohistochemistry analyses revealed that tumours from LV-miR145-SW620 cells had reduced Fascin-1 levels compared with LV-miRcontrol-SW620 cells (Figure 7B). Examination of the lungs clearly revealed that the number of mice with lung metastases was lower in the group implanted with SW620 cells expressing miR-145 compared with the group implanted with control SW620 cells (Figure 7C). These observations were consistent with our in vitro results.

**DISCUSSION**

In this study, the qPCR validation results showed that miR-145 was significantly downregulated in CRC cell lines, SW480, SW620, and LoVo, and moderately downregulated in HT2 and LS174T compared with the normal colon epithelial cell lines, FHC and NCM460. MiR-145 expression was significantly decreased in CRC tissues compared with the paired adjacent normal tissues, in which the mean expression of the miR-145 presented in CRC tissue samples was not completely incorporated in that of CRC cell lines. In addition, the downregulation of miR-145 was greater in lymph node and liver metastases than in primary CRC tumours. Statistical analyses revealed that miR-145 downregulation was significantly correlated with tumour progression in CRC patients. Furthermore, ectopically expressed miR-145 inhibited cell growth, migration, and invasion without significantly affecting cell cycle distribution in SW620 and LoVo cells; whereas, knockdown of endogenous miR-145 significantly enhanced these effects. By using a luciferase reporter assay, miR-145 was shown to suppress cell migration and invasion, and appeared to be associated with silencing of Fascin-1.
Regulatory analysis further confirmed that, as a negative regulator of Fascin-1, miR-145 directly targeted Fascin-1, resulting in decreased Fascin-1 mRNA and protein levels, both in vitro and in clinical specimens. On the basis of these findings, we hypothesise that miR-145 directly regulates Fascin-1, and that Fascin-1 has oncogenic activity in CRC and may be involved in aggressive behaviour of CRC. In addition, loss of miR-145, which is an endogenous Fascin-1 inhibitor, may promote aberrant expression of Fascin-1, increasing its abundance and contributing to the invasive and viability properties of CRC. In summary, these data suggested that miR-145 could be exploited for designing novel strategies against CRC metastasis in the future.
Most deaths from cancer are caused by complications arising from metastasis; therefore, targeting metastatic disease might be a potential anticancer strategy. To date, studies on tumour invasion and metastasis have revealed that miRNAs have a vital role in negatively regulating oncogenes and tumour suppressors (Lim et al., 2005; Dalmay and Edwards, 2006). For example, miR-9 directly represses the anti-metastatic gene E-cadherin, leading to tumour invasion and metastasis (Ma et al., 2010). Similarly, miR-183 represses osteosarcoma invasion and metastasis, in part by regulating Ezrin (Zhu et al., 2012). In CRC, there have been several studies examining the expression patterns of miRNAs and its role in invasion and metastasis (Asangani et al., 2008). By targeting PDCD4, miR-21 has been demonstrated to enhance CRC cellular invasion, intravasation, and metastasis; and miR-135a was found to promote growth and invasion of CRC by targeting metastasis suppressor 1 in vitro (Zhou et al., 2012). In addition, novel miRNAs were identified in clinical CRC samples by serial analysis of gene expression, which showed that miR-145 was steadily downregulated.
throughout CRC succession, from adenomatous to cancer, by these deregulated miRNAs (Cummins et al, 2006). Consistent with these reports, our results showed that miR-145 downregulation was greater in metastases than in primary CRC tissue, indicating a potential role for miR-145 in tumour invasion and metastasis.

Clinically, Fascin-1 has a central role in the invasive phenotype of several carcinomas (Hashimoto et al, 2005) and its protein has recently been proposed as a novel biomarker for aggressive tumour behaviour (Hashimoto et al, 2005). In agreement, our study showed an increased level of Fascin-1 in more metastatic CRC compared with less metastatic CRC, but the precise mechanism of Fascin-1-mediated metastasis is still unknown. By analysing miR-145 expression levels in different CRC cell lines in this present study, miR-145 was shown to be expressed at low levels in high-invasive cells and at high levels in low-invasive cells; conversely, Fascin-1 protein levels displayed the opposite expression pattern. It is probable that the upregulation of Fascin-1 by suppression of miR-145 contributed to tumour progression in CRC. Fascin-1 regulation by miR-145 was also examined in CRC cell lines by western blotting and the luciferase reporter assay. Paradoxically, it was found that in CRC cell lines, the specific sites targeted by miR-145 were not consistent with previous reports from oesophageal squamous cell carcinoma (Kano et al, 2010) and bladder cancer (Chiyomaru et al, 2010), in which miR-145 interacted with two putative miR-145 sites at positions 377–383 and 1140–1146. In our study, luminescence intensity was significantly decreased in miR-145 transfectants with two putative miR-145 sites at positions 116–122 and 377–383. As a result, we speculate that there are other target genes that interact competitively with miR-145 in CRC cell lines. These will need further investigation.

In agreement with previous reports showing an inhibitory effect of miR-145 (Sachdeva and Mo, 2012) on tumour growth, a growth reduction of SW620 cells upon transient miR-145 transfection was observed. This implied that miR-145 possessed a tumour suppressor function both in vitro and in vivo. On the basis of these findings, we suggest that miR-145 may have a role in the modulation of CRC metastases, acting as a metastamir. Our data implicate Fascin-1 in progression of CRC to a more invasive phenotype; therefore, the restoration of miR-145 expression could have important implications for clinical management of CRC. Although it is not yet clear whether Fascin-1 is itself subject to miRNA regulation, our study has established post-transcriptional regulation of Fascin-1 by miR-145.

In conclusion, miR-145 was found to suppress the invasion and metastasis of CRC by functioning as a tumour suppressor through the direct repression of Fascin-1. It may have potential therapeutic value in the treatment of CRC.

ACKNOWLEDGEMENTS

This work was supported by grants from the Natural Sciences Foundation of China (81071207, 81271622 and 31271053) and the 973 Program (2010CB912201 and 2010CB529904).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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