MiR-198 represses tumor growth and metastasis in colorectal cancer by targeting fucosyl transferase 8

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In this study we investigated the biological role and mechanism of miR-198 in colorectal carcinoma (CRC). MiR-198 expression was shown to exhibit a strongly negative correlation with lymph node invasion, distant metastasis and patient survival in examinations of colorectal cancer tissues and paired normal colorectal mucosa tissues. fucosyl transferase 8 (FUT8) was identified as a potential target of miR-198 in bioinformatics analysis and luciferase reporter assays. Overexpression of miR-198 in CRC cell lines decreased FUT8 levels as shown by immunofluorescence analysis, and inhibited cell proliferation, migration, and invasion. These anti-tumor phenotypes were rescued by reconstitution of FUT8 expression. Furthermore, miR-198 was shown to target the 3' UTR of FUT8 directly to downregulate FUT8 expression at both mRNA and protein levels in qRT-PCR and Western blot analyses, respectively. In vivo, restoration of miR-198 significantly inhibited xenograft growth and invasion of CRC tumors in nude mice. Therefore, it could be concluded that miR-198 suppresses the proliferation and invasion of CRC by directly targeting FUT8.

Colorectal cancer (CRC) is the third most common malignancies worldwide1. In China, the incidence and mortality rate of CRC is continually increasing2. Elucidation of the sequential progression from adenoma to carcinoma provides insights into strategies for the prevention and cure of CRC based on interference with this process; however, little improvement in the 5-year relative survival rate of CRC has been achieved, from 60% in 1987–1989 to 65% in 2003–2009 in United States1. Metastatic disease is the primary cause of death in CRC patients; therefore, elucidation of the molecular mechanisms underlying CRC metastasis and the key factors involved in this process is essential for the development of diagnostic and treatment strategies.

Tumor metastasis occurs via a series of discrete biological processes that move tumor cells from the primary neoplasm to a distant location. The metastatic cascade is a complex process that includes five steps: altered adhesion (invasion); intravasation; survival in the circulation; extravasation, and seeding at a distant site (metastatic colonization). Complex pathways and redundant molecules related to tumor metastasis, although identified, cannot fully explain the phenomenon of cancer metastasis. As microRNAs are now recognized as master regulators of gene networks and play important roles in tumourigenesis, it is no surprise that microRNAs have recently been demonstrated to have central roles during metastasis5. MicroRNAs, which are short (approximately 22 nucleotides) noncoding RNA molecules, guide the effector complex to bind with partially complementary sequences, usually within the 3' UTR of mRNAs, and inhibit protein synthesis with or without transcript degradation6. Accumulating evidence suggests an important role for a number of miRNAs in CRC metastasis, including miR-449b7, miR-211, miR-14114, miR-133a10, miR-62511. To date, downregulation of miR-198 has been found in many cancers, such as prostate cancer12, hepatocellular carcinoma13, lung cancer14, pancreatic cancer15, while its overexpression has been detected in esophageal cancer16, retinoblastoma17, and high grade prostate tumors18. More importantly, miR-198 shows an apparent anti-migratory effect in non-healing chronic diabetic ulcers by inhibiting keratinocyte migration, re-epithelialization and wound healing19. However, the specific role of miR-198 in CRC metastasis and the underlying mechanism remain to be elucidated.

In this study, we investigated the regulation of miR-198 in CRC by examining its expression in precancerous and cancerous tissues samples; its effects on cell growth, cell migration and invasion; and its role in CRC...
development and metastasis in a murine xenograft model. Furthermore, the mechanism(s) underlying the function of miR-198 in CRC was investigated with the aim of clarifying the correlation between miR-198 expression and the development and progression of colorectal cancer.

Results

MiR-198 expression correlates with colorectal cancer severity. The miR-198 expression level was firstly investigated in normal colorectal tissues and CRC tissues in a group of 116 CRC patients by ISH and another group of 65 CRC patients by quantitative reverse transcription PCR (qRT-PCR). MiR-198 expression was significantly decreased in CRC tissues compared with normal colorectal tissues (ISH, P < 0.0001, two-sided Student’s t-test, Figure 1 A, B; qRT-PCR, P < 0.001, Figure 1C). Furthermore, the correlation of miR-198 expression with different clinicopathological features of CRC was also investigated. Samples with downregulated miR-198 expression detected by ISH showed a significant association with histological grade (P = 0.003), tumor status (P = 0.01), AJCC stage (P = 0.002) and lymph node invasion (P = 0.004, Table 1). Interestingly, patients with low miR-198 expression exhibited significantly shorter survival time (P = 0.01, 7-year follow-up, Kaplan–Meier test, Fig. 1D). The prognostic significance of miR-198 expression was validated in this dataset by qRT-PCR. A consistent correlation of samples expressing low levels of miR-198 with lymph node invasion (P < 0.05) and distal metastasis status (P < 0.05, Supplementary Table 1) was observed. Taken together, these results demonstrate a close association between miR-198 downregulation and poor CRC prognosis, and further support our hypothesis that miR-198 functions as a tumor suppressor in CRC development.

MiR-198 inhibits CRC cell proliferation, migration and invasion in vitro. In light of the findings described previously, we speculated that miR-198 expression may inhibit malignant phenotypes of colorectal cells. To test this hypothesis, four stably transfected monoclonal cell lines were generated: two miR-198 overexpression stable cell lines (HCT116/miR-198 and SW1116/miR-198) and two empty vector transfected stable cell lines (HCT116/NC and SW1116/NC). Cell counting kit-8 (CCK-8) assays indicated that overexpression of miR-198 inhibits the cell proliferation rate in both SW1116 and HCT116 cells (Fig. 2 A, B). Furthermore, transwell and wound healing assays were used to monitor the effect of manipulating miR-198 expression on cell migration and invasion. Overexpression of miR-198 significantly decreased the number of SW1116 and HCT116 cells that penetrated the transwell filter, which suggested a substantial loss of cell migration and invasion ability in SW1116 (Fig. 2C, D) and HCT116 cells (Fig. 2E, F). Wound healing assays demonstrated that miR-198 expression significantly reduced the proliferation and migration of SW1116 and HCT116 cells (P < 0.01; Fig. 2G, H).

MiR-198 directly inhibits FUT8 expression in CRC cells. The predicted 3’UTR binding site was analyzed to identify the target genes of miR-198 in three independent online databases: PicTar, TargetScan, and miRDB, with a particular emphasis on those genes correlating with cancer metastasis. Overlap analysis revealed FUT8 as the only predictor targeting region of FUT8 3’UTR binding has miR-198 at the position 688–695 (Fig. 3A). Western blot analysis demonstrated that miR-198 overexpression significantly decreased FUT8 protein levels in SW1116 and HCT116 cells (P < 0.01; Fig. 3B). Furthermore, following cloning of the FUT8 promoter sequence into a luciferase reporter, miR-198 overexpression significantly reduced the FUT8 promoter luciferase activity. This effect was completely abolished after the putative miR-198-binding sites were mutated in both SW1116 and HCT116 cells (P < 0.01, Fig. 3C). Taken together, these data provide strong evidence that FUT8 is a specific target of miR-198 in colorectal cancer cells. The physiological occurrence of miR-198-induced FUT8 expression in CRC was analyzed in a group of 65 CRC tissue samples by qRT-PCR. Results revealed that FUT8 expression was negatively associated with miR-198 expression in CRC tissues (P = 0.01, paired t-test, Fig. 3D), indicating a key role for miR-198 in CRC.

FUT8 may be involved in miR-198-mediated cell behavior. Rescue experiments were performed to confirm that miR-198 modulates the biological behavior of colorectal cancer cells by repressing FUT8 expression. MiR-198 overexpression stable cell lines (HCT116/miR-198 and SW1116/miR-198) and control cells (HCT116/NC and SW1116/NC) were transfected with the FUT8 overexpression vector or control vector. CCK8 assays showed that overexpression of FUT8 rescued miR-198-mediated inhibition of cell proliferation (Fig. 4A, B). Furthermore, migration and invasion transwell assays (Fig. 4C, D, E, F) and wound healing assays of SW1116 cell lines (Fig. 4G, H) and HCT116 cell lines (Fig. 4J, K) showed that the miR-198-mediated inhibition of these processes was also abolished by FUT8 overexpression in these cell lines. These results indicate that miR-198 inhibits colorectal cancer cell proliferation, migration and invasion in vitro by repressing FUT8 expression.

MiR-198 suppresses FUT8 expression and inhibits tumor growth and metastasis in nude mice. To confirm the impact of miR-198 on FUT8 expression in vivo and to explore the possibility of targeting miR-198 as a therapeutic measure to suppress CRC, an SW1116 xenograft tumor model was established in BALB/C nude mice. The stably transfected human CRC SW1116 cells (SW1116/miR-198 or SW1116/NC) were implanted subcutaneously into athymic nude mice to allow tumor formation. At three weeks post- injection, the xenograft tumor volumes were significantly smaller in the miR-198 group compared with those in the control group (P < 0.001, Fig. 5A, B, C), indicating that miR-198 overexpression suppresses CRC tumor growth in vivo. Overexpression of miR-198 also substantially decreased the mRNA and protein level of FUT8 in xenograft tumors (Fig. 5D, E, F), which further supported our hypothesis that miR-198 suppresses FUT8 expression in vivo.

Finally, to investigate the role of miR-198 in CRC metastasis in vivo, five pairs of mice were sacrificed 8 weeks after implantation and liver was isolated and examined for the number of metastatic tumors. MiR-198 overexpression significantly decreased the metastasis in the liver compared with the control (Fig. 5G, H), indicating that miR-198 overexpression suppresses CRC metastasis in vivo. Collectively, our data indicate that miR-198 suppresses FUT8 expression and exerted an inhibitory function on human CRC growth and metastasis.

Discussion

Recent evidence has shown that miR-198 is involved in the pathogenesis of many human diseases. For example, unstable angina pectoris patients could be discriminated from stable patients based on their relatively high expression levels of a cluster of three microRNAs including miR-134, miR-198, and miR-37020. MiR-198 is also significantly overexpressed in preeclampsia22, and upregulated in the tissues of fetuses with anencephaly22. In cancer research, increasing bioinformatics evidence and subsequent analysis in functional assays has indicated that miR-198 is involved in many steps during cancer progression, such as apoptosis, proliferation, migration and metastasis.

It has been reported that several genes can be targeted by miR-198 in different type of cancers and miR-198 has different functions during cancer progression21,23,24. Tan et al. showed that miR-198 diminishes hepatocyte growth factor (HGF) stimulated migration and invasion of hepatocellular carcinoma cell negatively by modu-
lates c-MET expression, suggesting miR-198 could serve as a novel suppressor of hepatocellular carcinoma cells migration and invasion by negative regulating the HGF/c-MET pathway. And in our study, we also found that the expression of c-MET after miR-198 overexpression was down-regulated in colorectal cancer cell lines SW1116 and HCT116 (data not shown). Besides, Elfimova found that miR-198 acts as a tumor suppressor by repression of migogenic and mogogenic pathways diminishing hepatocellular carcinoma cell growth and migration. Ye et al. discovered that miR-198 is associated with cell apoptosis by mediating repression of livin expression in prostate cancer. A recent study found that miR-198 can impair human lung cell proliferation by targeting FGFR1 and then leads to...
Inhibition of lung cancer development\(^2\). Taken together, these results suggest that the effects of miR-198 are highly dependent on its target in each type of cancer.

The pathological role and the regulatory mechanism of miR-198 in the development and progression of colorectal cancer, however, remain unclear. In the present study, we demonstrated that miR-198 suppressed the proliferation and invasion of CRC, thus providing evidence that miR-198 may contribute to colorectal carcinogenesis by targeting *fucosyl transferase 8* (*FUT8*).

In this study, miR-198 was shown to be downregulated in colorectal cancer, and its down-regulation significantly correlated with more lymph node invasion, more distant metastasis and shorter patient survival. These clinicopathological findings suggested a scenario in which miR-198 contributes to the anti-tumor phenotypes of colorectal cancers. This hypothesis is supported by both *in vitro* and *in vivo* evidence. In cultured colorectal cancer cells, overexpression of miR-198 resulted in significant growth inhibition, and loss of migration and invasiveness. In xenograft mouse models, restoration of miR-198 significantly delayed xenograft growth and invasion of CRC tumors. These findings consistently indicated that miR-198 overexpression represses the aggressiveness of colorectal cancer cells, thereby explaining the depletion of miR-198 found in advanced colorectal cancers. To date, no reports have described such a relationship between miR-198 and CRC. Furthermore, its functions in the development of colorectal cancer, especially on cell proliferation and invasion, have not been reported previously.

More importantly, miR-198 was shown to function by directly targeting *FUT8* in colorectal cancer proliferation and metastasis. *FUT8* is the only enzyme responsible for α1,6-linked (core) fucosylation by adding fucose to the innermost GlcNAc residue of a N-linked glycan in mammals. A growing body of evidence indicates that core fucosylation is important for regulating protein functions. For instance, the increase in core fucosylation has been shown to strengthen cell-cell adhesion in human colon carcinoma cells\(^3\). The upregulation of *FUT8* mRNA, protein, and activity has been observed in several malignant tumors including ovarian\(^4\), thyroid\(^5\), lung\(^6\) and colorectal\(^6\) cancers, and has been linked to the severity of cancers.

Predicted by bioinformatics analysis and validated by luciferase reporter gene assay, *FUT8* was identified in this study as the major regulatory target of miR-198. The strong effect of miR-198 on *FUT8* was systemically validated in cultured cells and an *in vivo* xenograft mouse models. In colorectal cancer cells, miR-198 displayed a strong inhibitory effect on *FUT8* expression. Consistent with this observation, overexpression of miR-198 in xenograft mouse models also dramatically decreased *FUT8* expression levels, and a significantly negative correlation was observed between *FUT8* and miR-198 expression in human CRC tissues. Interestingly, we also discovered that the anti-tumor phenotypes of miR-198 in CRC can be partly rescued by reconstitution of *FUT8* expression. In addition to *FUT8*, many genes relevant for the metastatic phenotype were predicted to be targeted by miR-198. Further investigations are necessary to identify and characterize other miR-198 targets to fully elucidate the functional role of miR-198 in colorectal cancer development, progression and metastasis.

The mechanisms underlying miRNA dysregulation in malignant tissues have been studied by several groups. It has been reported that the miRNA expression is regulated by different signaling pathways in a cell context-dependent manner. For example, the transforming growth factor-beta pathway, which is commonly inactivated in the late stages of CRC, has been reported to suppress miR-29a expression in the fibrotic process\(^7\). The canonical WNT signaling pathway induces miR-29a expression in osteoclast cells via the TCF4/LEF1-binding sites in the proximal promoter of miR-29a, whereas miR-29a directly suppresses WNT antagonist expression to form a positive feedback loop for WNT signaling\(^8\). Recent advances in the field of epigenetics have revealed that microRNAs’ expression is also under epigenetic regulation\(^9\). Tsuruta et al. reported that miR-152, a tumor suppressor miRNA, is silenced by DNA hypermethylation in endometrial cancer\(^9\). The significance of both signaling pathways and epigenetics has been reported in the development, progression and metastasis of colorectal cancer; therefore, exploration of the mechanism of miR-198 dysregulation in CRC is warranted in future studies.

In conclusion, this study has provided the first evidence that miR-198 expression is decreased in colorectal cancer and its down-regulation significantly associated with severity of CRC. Furthermore, our study shed light to the importance of miR-198 in suppressing CRC cell proliferation and metastasis by directly targeting *FUT8*.

Methods

Ethics statement. All animal experiments were performed in accordance with the guidelines of China Council on Animal Care and Use. All animal procedures carried out in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee of the Ethics Committee of Shanghai Jiao Tong University School of Medicine, China. All animals were kept and the experiments were performed in accordance with the European Community guidelines for the use of experimental animals (86/609/EEC).

Patients and CRC biopsy specimens. A total of 65 pathologically confirmed CRC patients were enrolled into this study and underwent surgery at the Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University (China), between December 2009 and November 2012. The study was approved by the ethics committee of Shanghai Jiao Tong University School of Medicine, and written informed consent was obtained from all patients at study entry. Two tissue microarrays, including 116 pairs of CRC and corresponding non-tumor tissues were purchased from BioChip (Shanghai, China).

RNA isolation, reverse transcription and quantitative real-time PCR analysis. Total RNA was extracted from 65 pairs of matched human CRC specimens (including cancerous and adjacent non-tumor tissues) using Trizol reagent (Invitrogen, Carlsbad, CA, USA). All tissue samples were examined histologically, and all matched
Figure 2 | Overexpression of miR-198 inhibits colorectal cancer cell proliferation, migration and invasion in vitro. SW116 and HCT116 cells were stably transfected with a miR-198 overexpression plasmid or control negative plasmid for subsequent analysis. Data in all statistical plots represent means ± SD. (A,B) Proliferation curves of SW116 and HCT116 cells, respectively, as determined by CCK-8 assay. The rate of proliferation of the miR-198 overexpression cell lines was significantly inhibited compared with that of the control cells (P < 0.001, two-sided t-test). (C,D,E,F) Cell migration (at 24 h and 36 h) and invasion ability (at 48 h and 60 h) in SW116 (C,D) and HCT116 (E,F) cells were analyzed in transwell assays. Migrated cells and invaded cells stained by 0.1% crystal violet were counted in 8 randomly microscope fields (×200). Statistical analyses are based on three independent experiments and representative images are shown. MiR-198 overexpression reduced the migration and invasion ability of both cell lines compared with the negative controls. (G, H) Wound healing assays demonstrated that miR-198 expression significantly reduced the proliferation and migration of SW116 and HCT116 cells (P < 0.01). Statistical analysis is based on three independent experiments and representative images are shown.
non-tumor tissues (taken at least 5 cm from the tumor) were confirmed to be normal. Reverse transcription was performed using the First Strand cDNA Synthesis Kit (Takara, Shiga, Japan), according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq II (Takara, Shiga, Japan). To quantitate miR-198 expression, total RNA was polyadenylated and reverse transcribed using an All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA), according to the manufacturer's protocol. The expression of each target gene was normalized to that of 18S or U6 snRNA. Quantification was calculated using the 2^(-ΔΔCT) method and is presented as fold change. Primer (Sangon, Shanghai, China) sequences were: FUT8 (forward, 5′-AACTGGTTCAGCGGAGAATAAC-3′; reverse, 5′-TGAGATTCCAAGATGTTGTCGG-3′); 18S (forward, 5′-CGGACAGGATTGACAGATTGATAGC-3′, reverse, 5′-TGCCAGAGTCTCGTTCGTTATCG-3′).

Figure 3 | FUT8 is the specific target of miR-198 in colorectal cancer cells and has a negative correlation with miR-198 expression in CRC tissues. SW1116 and HCT116 cells were stably transfected with a miR-198 overexpression plasmid or control negative plasmid for subsequent analysis. Data in all statistical plots represent means ± SD. (A) Overlap analysis revealed FUT8 in all the three independent online databases: PicTar, TargetScan, and miRDB. (B) Representative Western blot and analyzed data showing that miR-198 overexpression significantly decreases total FUT8 expression in SW1116 and HCT116 cells. Statistical analysis is based on three independent experiments which have been run under the same experimental conditions and representative Western blot images are shown. (C) Luciferase reporter assays suggest that miR-198 inhibits FUT8 promoter activity but not the mutant sequence devoid of the putative binding sites. The luciferase activity is calculated as the ratio of firefly (reporter)/renilla (normalization) signals. (D) qRT-PCR analysis reveals a negative correlation between miR-198 and FUT8 mRNA expression in CRC tissues (P = 0.01, paired t-test).
Figure 4 | FUT8 may be involved in miR-198-mediated cell behavior. Fucosyl transferase 8 (FUT8) overexpression rescues miR-198-mediated inhibition of colorectal cancer cell proliferation, migration and invasion in vitro. SW1116 and HCT116 cells were stably transfected with a miR-198 overexpression plasmid or control negative plasmid and analyzed in CCK8 assays (A,B), transwell migration and invasion assays (C,D,E,F) and wound healing assays (G,H,J,K). Images are of representative fields for each experiment. Data in all statistical plots represent means ± SD.
In situ hybridization analysis of miR-198. A full length DIG-labeled locked nucleic acid (LNA) probe ((Exiqon, Vedbæk, Denmark)) specific for miR-198 snRNA was prepared. In addition, a probe specific for U6 snRNA was used as a positive control, and a 22-mer scrambled probe with a random sequence having no known complementary sequence was included as negative control. In situ hybridization (ISH) analysis of the CRC and corresponding non-tumor tissue microarrays was performed with a Tecan Freedom Evo automated hybridization instrument (Tecan, Männedorf, Switzerland) as follows: proteinase-K treatment (15 µg/ml) at 37°C for

Figure 5 | MiR-198 suppresses FUT8 expression and inhibits tumor growth and metastasis in nude mice. (A,B) Overexpression of miR-198 inhibits growth of xenografts in nude mice. Stably transfected human CRC SW1116 cells (SW1116/miR-198 or SW1116/NC) were injected subcutaneously into the right forelimb axilla to establish a xenograft model. The sizes of in-situ and resected tumors arising from cells transfected with/without the miR-198 overexpression plasmid (at 21 days post-injection) are shown in panels (A) and (B), respectively. (C) Tumor growth curves in the miR-198 overexpression and control groups. MiR-198 overexpression significantly suppressed xenograft tumor growth ($P < 0.001$, t-test of tumor sizes). (D,E,F) qRT-PCR(D), Western blotting (E) and immunofluorescence (F) analyses show that overexpression of miR-198 dramatically decreases the FUT8 mRNA and protein levels in xenograft CRC tumors. Statistical analysis is based on three independent experiments which have been run under the same experimental conditions. Representative immunofluorescence images are shown and statistical results are indicated. (G,H) Gross and microscopic examinations (G) and occurrence (H) of liver metastasis in the miR-198 overexpression and control groups. Arrow and the letter M indicate metastasis.
Plasmid construction and stable transfection. The pGFP-miR-198 plasmid containing the miR-198 coding sequence was purchased from Shanghai GeneChem (China) and verified by DNA sequencing. The plasmid encodes a red-shifted variant of wild-type green fluorescent protein (GFP) that has been optimized for brighter fluorescence and greater expression in mammalian cells. For plasmid transfection, HCT116 and SW1116 cells were seeded into 6-well plates 24 h before transfection with plasmids (4 μg/well) using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. The pGFP-miR-NC construct was used as an empty vector control. Transfected HCT116 and SW1116 cells were selected using G418 (500 μg/ml and 800 μg/ml, respectively) to generate four stably transfected monoclonal cell lines: two miR-198 overexpression stable cell lines (HCT116/miR-198 and SW1116/miR-198) and two control stable cell lines (HCT116/NC and SW1116/NC).

Cell migration and invasion assays. Transwell assays were performed in 6.5-mm diameter Boyden chambers with pore size of 8.0 μm (Corning, NY, USA).

For migration assays, the stable monoclonal cell lines (HCT116/miR-198, SW1116/miR-198, HCT116/NC and SW1116/NC) and these stable cell lines transfected with FUT8 plasmid or negative control plasmid (1.5 × 10⁴ cells per well) were resuspended in the migration medium (medium without FBS) and placed in the upper compartment of the transwell chamber with a 0.8-μm transwell filter. The lower compartment was filled with 600 μl medium containing 30% FBS as a chemoattractant. After incubation for 24 h at 37 °C with 5% CO₂ in a humidified incubator, the transwell filters were removed and the filters were mounted on a light microscope (Carl Zeiss, Germany). The width of the wound area was measured using NIH Image J analysis and normalized to the time 0 wound for four independent experiments.

For invasion assays, cell invasion (3.0 × 10⁴ cells per well) was measured in 24-well matrigel-coated invasion chambers over an incubation period of 48 h.

Wound healing assay. The stable monoclonal cell lines (HCT116/miR-198, SW1116/miR-198, HCT116/NC and SW1116/NC) and these stable cell lines transfected with FUT8 plasmid or negative control plasmid (5.0 × 10⁴ cells per well) were seeded into 6-well plates (Corning, NY, USA) 24 h before growing to confluence. Injured scratch wounds were then created (in triplicate) on the confluent monolayer using a pipette tip and cells were cultured in the medium without FBS. Immediately after wounding (time 0) and at 48 h, images were taken using a digital camera mounted on a light microscope (Carl Zeiss, Germany). The width of the wound gap was measured using NIH Image J analysis and normalized to the time 0 wound for four independent experiments.

Cell proliferation assay. The stably transfected monoclonal cell lines (HCT116/miR-198, SW1116/miR-198, HCT116/NC and SW1116/NC) and these stable cell lines transfected with FUT8 plasmid or negative control plasmid (3.0 × 10⁵ cells per well) were plated into triplicate wells of a flat-bottomed 96-well plate (Corning, NY, USA). Cell proliferation was assessed after 24, 48, 72 and 96 h using the cell Counting Kit-8 (Dojindo, Tokyo, Japan) method according to the manufacturer’s instructions.

Bioinformatics and luciferase assay. Potential microRNA targets were predicted and analyzed using three publicly available algorithms: PicTar, TargetScan, and miRanda. The number of false positive results was minimized by accepting only putative target genes that were predicted by at least two programs. The 3′ UTR of target genes was amplified from human genomic DNA and cloned into the pGL3-control vector (Promega, Madison, WI, USA). Site-directed mutagenesis of the miR-198 target-site in the target gene 3′ UTR was carried out using a site-directed mutagenesis kit (Takara, Shiga, Japan). The stably transfected monoclonal cell lines (HCT116/miR-198, SW1116/miR-198) were seeded into 12-well plates (Corning, NY, USA) and transfected after 24 h with 1 μg of wild-type (wt) 3′ UTR FUT8 vector, mutant (mt) 3′ UTR FUT8 vector or the empty control vector using the Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. After incubation for 48 h, cells were assayed using the Dual-Luciferase reporter assay system kit (Promega, Fitchburg, WI, USA), according to the manufacturer’s instructions. All experiments were performed in triplicate and data were pooled from three independent experiments.

Western blotting. Whole cell lysates were prepared from the cancer cell lines and standard Western blotting analysis was performed using anti-FUT8 (mouse polyclonal antibody, Alcam, UK) and anti-GAPDH (Kangchen Biotechnology, China) antibodies. All primary antibodies were used at a 1:1,000 dilution. Peroxidase-conjugated anti-goat or anti-mouse IgG secondary antibodies were obtained from Kangchen Biotechnology (China) and used at a 1:5,000 dilution. Three independent experiments were performed using Student’s paired t-test, spearman’s correlation test, or the Chi square test; P < 0.05 was considered statistically significant.
