Cluster microRNAs miR-194 and miR-215 suppress the tumorigenicity of intestinal tumor organoids

Toshiaki Nakaoka,1 Yoshimasa Saito,1 Yuiko Shimamoto,1 Toshihide Muramatsu,1 Masaki Kimura,1 Yae Kanai2 and Hidetsugu Saito1

1Division of Pharmacotherapeutics, Keio University Faculty of Pharmacy, Minato-ku, Tokyo; 2Department of Pathology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

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Correspondence
Yoshimasa Saito, Division of Pharmacotherapeutics, Keio University Faculty of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan.
Tel/Fax: +81-3-5400-2692; E-mail: saito-ys@pha.keio.ac.jp

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MicroRNA (miRNA) are 21–25-nucleotide non-coding RNA that can post-transcriptionally downregulate the expression of various target genes. miRNA are expressed in a tissue-specific manner and play important roles in cell proliferation, apoptosis and differentiation during mammalian development.1–3 Misexpression of cancer-related miRNA leads to the initiation and progression of cancer through modulation of their target oncogenes or tumor suppressor genes.4,5 We have reported that some important tumor suppressor miRNA are silenced by epigenetic alterations such as DNA methylation and histone modification in human cancer cells.6,7,8

Accumulated evidence has clarified that cancer cells in solid cancer tissues are heterogeneous, showing a hierarchy of stemness.9,10 Stem cells have the ability to perpetuate themselves through self-renewal and to generate mature cells of various tissues through differentiation.11 A subpopulation of cancer cells with distinct stem-like properties is responsible for tumor initiation, invasive growth and metastasis formation, and these are defined as cancer stem cells (CSC). As CSC are considered to be resistant to conventional chemotherapies and radiation therapy, it would be desirable to develop a therapeutic strategy specifically targeting CSC.

Lgr5, a member of the Wnt signaling pathway, has been identified as a new molecular marker of stem cells in the small intestine, colon, stomach, liver and pancreas.7–12 The newly developed technique of 3D culture allows Lgr5-positive stem cells to form budding cyst-like structures (organoids) that resemble the properties of original tissues.7 This type of 3D culture uses serum-free medium that includes only predefined factors such as R-spondin 1 (Rspo1) and epidermal growth factor (EGF). Rspo1 has been identified as a ligand for Lgr5 and is an essential factor for activation of the Wnt signaling pathway.13,14 Thus, interaction between Lgr5 and Rspo1 plays a pivotal role in the self-renewal of stem cells. Using this new 3D culture system, organoid models of human colon, prostate and pancreatic cancers have been established, and these have thrown light on the biology of cancer stem cells.10,15–17

Here we established organoids derived from intestinal tumors of Apcmin/+ (Min) mice and normal intestinal epithelia of C57BL/6J mice. The organoid culture system allowed expansion of Lgr5-positive stem cells into cyst-like structures with properties resembling the original tissues. Thus, organoids derived from tumors of Min mice are considered to be a desirable model for the initial stage of intestinal tumorigenesis according to the adenoma-carcinoma sequence.18 In the present study, we investigated the roles of miRNA relevant to the initial stage of tumorigenesis in intestinal tumor organoids.

Materials and Methods
Organoid culture. We performed organoid culture of intestinal tumors of Min mice and normal epithelia from wild-type C57BL/6 mice. In brief, isolated tumor cells and epithelial cells were embedded in Matrigel on ice (growth factor-reduced, phenol red-free; BD Biosciences, San Jose, CA, USA) and seeded in 48-well plates. The Matrigel was polymerized for 10 min at
37°C, and overlaid with 250 μL/well basal culture medium (advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, Glutamax, 1 × N2, 1 × B27 [all from Thermo Fisher Scientific, Waltham, MA, USA] and 1 mmol/L N-acetylcysteine [Sigma-Aldrich, St. Louis, MO, USA]) containing the following optimized growth factor combinations: EGF and noggin for intestinal tumors; and EGF, noggin and Rspo1 for intestinal epithelia. For drug treatment, cells were seeded 2 days before drug administration and treated with 10 nM irinotecan (Wako, Pure Chemical Industries, Osaka, Japan) for 48 h. All experiments and procedures were approved by the Keio University Animal Research Committee, and all methods were carried out in accordance with the approved guidelines.

Cell proliferation assay. Cell proliferation activity of organoids was examined by WST assay with a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer’s instructions.

Lentivirus-mediated overexpression of miR-194 and miR-215. Overexpression of miR-194 and miR-215 in tumor organoids was conducted using lentiviral vectors. A puromycin-resistant lentiviral vector (pLVsin-CMV Pur) was obtained from (Takara Bio, Shiga, Japan). The sequences of the probes conformed to the miRBase Release 19 (Toray, Kamakura, Japan). The annotation and oligonucleotide labeling kit (Toray Industries, Kamakura, Japan). Labeled RNA extracted from cancer cell lines using the mirVana miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA). Extracted total RNA was checked with a Bioanalyzer (Agilent Technologies, CA, USA) and labeled with a 3D-GenmiRNA labeling kit (Toray Industries, Kamakura, Japan). Labeled RNA were hybridized onto 3D-Gen Mouse miRNA Oligo chips (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase Release 19 database (www.mirbase.org). After stringent washes, the fluorescent signals were scanned with a 3D-Gen Scanner (Toray Industries) and analyzed using the 3D-Gen Extraction software (Toray Industries). The raw data for each spot was normalized by subtraction with the mean intensity of the background signal determined from the signal intensities of all blank spots with 95% confidence intervals. Measurements for spots with signal intensities greater than two standard deviations (SD) of the background signal intensity were considered to be valid. The relative expression level of a given miRNA was calculated by comparing the signal intensities of the valid spots throughout the microarray experiments. The normalized data were globally normalized per array, so that the median of the signal intensity was adjusted to 25. All data were submitted to the GEO database, under the accession number GSE75482.

Quantitative RT-PCR. Expression levels of genes were analyzed by TaqMan quantitative RT–PCR assay for miR-215, miR-194, miR-192 and Lgr5 (Applied Biosystems) in accordance with the manufacturer’s instructions. Expression levels for Mdm2, E2F3, Ctnnb1p and Ereg were analyzed by Universal SYBR Select Master in accordance with the manufacturer’s instructions. The primers are listed in Table S2. U6 was used as an internal control for miR-215, miR-194 and miR-192. Gapdh was used as an internal control for Lgr5, Mdm2, E2F3, Ctnnb1p and Ereg. All experiments were carried out in triplicate.

Western blotting. Western blotting was performed with antibodies against p53 (FL–393) and Lgr5 (Anti-GPCR GPR49 antibody) purchased from Santa Cruz Biotechnology and Abcam, respectively. HRP-conjugated β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an internal control. Quantitative analysis was performed using the Image-J.

Apoptosis assay. Apoptosis assay was performed using a GFP-Certified Apoptosis/Necrosis Detection Kit for microscopy and flow cytometry (Enzo Life Sciences, Farmingdale, NY, USA).

Cell cycle assay. Cells were harvested by trypsinization, washed with PBS and fixed in 70% ice-cold ethanol overnight at 4°C. They were then washed with PBS and treated with RNAe A (1 μg/ml) at 37°C for 60 min and incubated with propidium iodide (50 μg/ml) for 30 min at room temperature. After incubation, flow cytometry analysis was performed.

Patients and tissue specimens. Paired samples of primary colorectal cancer and corresponding non-tumor colorectal mucosa were obtained from surgically resected specimens of five patients treated at the National Cancer Center Hospital, Tokyo, Japan. These fresh frozen tissues were subjected to extraction of total RNA. The clinicopathological features of these patients, including age, sex, tumor location, tumor size, histologic type and TNM stage, are described in Table S3. All experiments and procedures were approved by the Ethics Committees of the National Cancer Center, and all methods were carried out in accordance with the approved guidelines. Written informed consent was obtained from all of the patients.

Luciferase assay. In brief, the 3’untranslated region (UTR) including miRNA target sites of the Mdm2, E2F3, Ctnnb1p and Ereg genes were amplified by PCR from mouse genomic DNA in accordance with previous reports(19–21) and TargetScan (http://www.targetscan.org). The luciferase constructs were made by ligating the 3’ UTR of the Mdm2, E2F3, Ctnnb1p and Ereg genes into the Xba I site of the pGL3-control vector (Promega, Madison, WI, USA). NIH3T3 cells were cultured in DMEM medium supplemented with 10% FBS. The cells were then transfected with the firefly luciferase reporter vector containing the 3’ UTR of the Mdm2, E2F3, Ctnnb1p and Ereg genes and the control vector containing Renilla luciferase pRL-CMV (Promega) using lipofectamine 3000 (Thermo Fisher Scientific) in six-well plates. The luciferase assays were performed 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega). The activity of firefly luciferase was normalized to that of Renilla luciferase.

Flow cytometry. Flow cytometry analysis was performed with the PE-conjugated mouse anti-Lgr5 monoclonal antibody (1:50, clone 2A2; OriGene, Rockville, MD, USA) using a BD LSR-II flow cytometer (BD Bioscience). PE-conjugated mouse IgG (R&D Systems, Minneapolis, MN, USA) was used as an isotype control.

Statistical analysis. All statistical analyses were performed using R studio. Dunnett’s test and Tukey’s test were used (**P < 0.05, ***P < 0.01, ****P < 0.001. All error bars represent SD).

Results

miR-194 and miR-215 are significantly downregulated in intestinal tumor organoids. Here we established cultured organoids...
from normal intestinal epithelia of wild-type mice and intestinal tumors of Min mice. Because mutation of the Apc gene is an early event in the multi-step carcinogenesis according to the adenoma-carcinoma sequence theory,18) Apc-deficient tumor organoids may be a desirable model for investigating the molecular mechanism underlying colorectal carcinogenesis.

As described previously, organoids derived from Apc-deficient tumors formed cystic organoid structures without the budding that is observed in organoids derived from normal intestinal epithelia. Because Apc loss constitutively activates the Wnt pathway, Rspo1 was dispensable for the culture of Apc-deficient tumor organoids (Fig. 1a). We dissected three Min mice, and established tumor organoids from two to three tumors of each mouse. We successfully established tumor organoids from all of the tumors examined, and confirmed that the organoids showed no morphological or biological changes.

We comprehensively examined the expression levels of 1265 miRNA transcripts by microarray analyses. This revealed that 55 miRNA were significantly upregulated and 35 miRNA were significantly downregulated in intestinal tumor organoids in comparison with normal epithelial organoids. miR-215 was the main miRNA downregulated in intestinal tumor organoids, in comparison with organoids derived from normal epithelia (Fig. 1b). miR-215 is located in the miRNA cluster with miR-194-1-5p on chromosome 19. miR-194-1-5p and miR-194-2 is also downregulated in intestinal tumor organoids, and the expression of both miRNAs is considered to alternate in a coordinated manner. To validate the microarray data, we performed quantitative RT-PCR of miR-215 and miR-194. The relative expression levels of miR-215 and miR-194 were significantly decreased in tumor organoids relative to normal organoids (Fig. 1c). Expression of miR-194 may be influenced by miR-194-2, which is located in the other miRNA cluster with miR-192 on chromosome 19. We examined the expression levels of miR-192 by quantitative RT-PCR. As shown in Figure 1(c), the expression level of miR-192 was also downregulated in tumor organoids relative to normal organoids, but the ratio (Tumor/Normal) of miR-192 expression was higher than those of miR-215 and miR-194. Therefore, we focused our study on miR-215 and miR-194.

We investigated the expression levels of miR-215 and miR-194 in human colon cancer tissues as well as non-tumor epithelial tissues obtained from colon cancer patients. As shown in Figure 1(d), the expression levels of miR-215 and miR-194 were lower in colon cancer tissues than in non-tumor epithelial tissues, consistent with the findings obtained using tumor and normal organoids derived from Min and wild-type mice.

Enforced expression of miR-194 suppresses the growth of intestinal tumor organoids. To investigate the function of miR-194 and miR-215, we overexpressed them in tumor organoids, in which these miRNA are markedly suppressed. We performed lentivirus-mediated overexpression of miR-194, miR-215 and the cluster containing both miR-194 and miR-215 in tumor organoids. As shown in Figure 2(a), significant overexpression of miR-194 and miR-215 was confirmed in tumor organoids, in which each miRNA was transfected. To examine the roles of miR-194 and miR-215 on cell growth, WST cell proliferation assays were performed using the miRNA-transfected tumor organoids. Significant inhibition of cell proliferation was observed in both miR-194 and cluster-transfected organoids, whereas overexpression of miR-215 did not significantly suppress cell growth (Fig. 2b). The inhibitory effect of cell proliferation was more profound in organoids overexpressing both miR-194 and miR-215 by induction of cluster transfection in organoids transfected with only miR-194 (Fig. 2b). The proliferative potential of organoids was analyzed in terms of their size (Fig. 2c,d). Figure 2(f) shows the size distribution of organoids normalized to the total number of colonies for each treatment. The tumor organoids transfected with miR-194 and cluster were smaller than those transfected with the empty vector (control) and miR-215 (Fig. 2c,d,f).

To clarify the molecular mechanism underlying cell growth suppression by miR-194, we examined the expression of Mdm2, which is a direct target of miR-194, and miR-215, a negative regulator of p53.22) As expected, we found that enforced expression of both miR-194 and miR-215 significantly repressed Mdm2 expression, resulting in upregulation of p53 (Fig. 3a). In addition, we investigated the relative...
Enforced expression of miR-215 suppresses the cancer stem cell signature. A recent study has shown that CDX1 directly activates miR-215 expression, and that miR-215 then represses the expression of stem cell genes and promotes cell differentiation via inhibition of stemness. Because organoids are formed from Lgr5-positive stem cells, the number of organoids is considered to be correlated with stemness. We investigated the effect of miR-215 on stemness by counting the number of tumor organoids transfected with each miRNA per well (Fig. 2c,e). As shown in Figure 2(e), the number of tumor organoids was significantly decreased by transfection of miR-215, whereas miR-194 had no effect on the number of tumor organoids. To investigate the molecular mechanism underlying inhibition of stemness by transfection of miR-215, we examined the expression levels of Lgr5, a newly identified stem cell marker, by quantitative RT-PCR and western blotting. The expression of Lgr5 mRNA and protein was significantly suppressed in tumor organoids transfected with miR-215 and the cluster, in comparison to control and tumor organoids transfected with miR-194 (Fig. 4a). In addition, we performed flow cytometry to determine the number of Lgr5-positive cells. As shown in Figure S1(a), we confirmed that Lgr5-positive cells were decreased in tumor organoids transfected with miR-215 and the cluster, in comparison to control and tumor organoids transfected with miR-194. These results are consistent with the findings of Western blotting and quantitative RT-PCR. We also examined the expression level of Ereg, which has been identified as a direct target of miR-215 and an intestinal cancer stem cell marker. We confirmed that Ereg was downregulated in tumor organoids transfected by miR-215 (Fig. 4a). The relative luciferase activity of the construct containing the target site of miR-215 in the 3' UTR of the Mdm2, E2f3 and Ctnnbip1 genes were significantly lower than those of the constructs lacking these regions. As shown in Figure S1(b), the expression levels of E2f3 and Ctnnbip1 genes showed that the relative luciferase activities of the constructs containing the target sites of miR-194 and miR-215 in the 3' UTR of the Mdm2, E2f3 and Ctnnbip1 genes were significantly lower than those of the constructs lacking these regions. Thus, enforced expression of miR-215 may lead to the growth arrest of tumor organoids via overexpression of p53 and an increase of G0/G1 phase cells. Because E2f3 and Ctnnbip1 play important roles in cell cycle and cell proliferation, we performed cell cycle and apoptosis assays using miRNA-transfected organoids. The cell cycle assay demonstrated that suppression of E2f3 by transfection of miR-194 and the cluster increased the G0/G1 phase, indicating cell cycle arrest. Flow cytometric analyses revealed that annexin V-positive cells were increased in cluster-transfected organoids, indicating that induction of both miR-194 and miR-215 caused cell apoptosis.
miR-194 and 215 suppress tumor organoids

To evaluate the effect of miR-215 on chemosensitivity, we treated miRNA-transfected organoids with irinotecan. The results of the cell proliferation assay revealed that transfection with miR-194 and the cluster caused significant growth inhibition in the tumor organoids, whereas after treatment with irinotecan (IC50), growth inhibition compared to the control was abrogated. In contrast, organoids transfected with miR-215 and the cluster showed considerably increased sensitivity to irinotecan, suggesting that miR-215 inhibits stemness and increases sensitivity to irinotecan (Fig. 4b).

Discussion

Decreased expression of miR-194 has been reported in various types of malignancy, including liver, lung, stomach and colorectal cancers, as well as multiple myeloma. Overexpression of miR-194 in gastrointestinal cancer cells suppresses cell migration, invasion and metastasis. These reports suggest that miR-194 functions as a tumor suppressor in various human malignancies. It has been reported that downregulation of miR-215 may be a prognostic marker in patients with colon cancer.

Here we report that the cluster miRNAs miR-194 and miR-215 are downregulated in intestinal tumor organoids, as well as in human colon cancer tissues. In particular, the expression level of miR-215 was very low in tumor organoids relative to normal epithelial organoids, whereas the observed differences

Fig. 3. Relative expression levels of target genes of miR-194 and miR-215 in intestinal tumor organoids. (a) Quantitative RT-PCR analysis of Mdm2 normalized to Gapdh and relative luciferase activities of the construct containing the target sites of miR-194 and miR-215 in the 3′ UTR of the Mdm2 gene and the constructs lacking these regions (upper). Quantitative analysis for western blotting of p53 normalized to β-actin in intestinal tumor organoids transfected with miR-194, miR-215 and the cluster (left). Relative luciferase activities of the construct containing the target site of miR-194 in the 3′ UTR of the Mdm2 gene and the constructs lacking this region (lower). (b) Quantitative RT-PCR analysis of E2f3 normalized to Gapdh in intestinal tumor organoids transfected with miR-194, miR-215 and the cluster (left). Relative luciferase activities of the construct containing the target site of miR-194 in the 3′ UTR of the E2f3 gene and the construct lacking this region (right). (c) Quantitative RT-PCR analysis of Cnnbip1 normalized to Gapdh in intestinal tumor organoids transfected with miR-194, miR-215 and the cluster (left). Relative luciferase activities of the construct containing the target site of miR-215 in the 3′ UTR of the Cnnbip1 gene and the construct lacking this region (right). (d) Cell cycle assay and apoptosis assay of intestinal tumor organoids transfected with miR-194, miR-215 and the cluster. *P < 0.05.

Fig. 4. miR-215 inhibits stemness of intestinal tumor organoids. (a) Quantitative RT-PCR analysis of Lgr5 normalized to Gapdh and quantitative analysis of western blotting of Lgr5 normalized to β-actin (upper). Quantitative RT-PCR analysis of Ereg normalized to Gapdh, and relative luciferase activities of the construct containing the target site of miR-215 in the 3′ UTR of the Ereg gene and the construct lacking this region (lower). (b) WST cell proliferation assay of miRNA-transfected intestinal tumor organoids with or without irinotecan treatment (10 nM: IC50). *P < 0.05, **P < 0.01.
between human colon cancer tissues and non-tumor tissues were not large. Tumor tissues contain many non-epithelial cells, such as stromal cells including fibroblasts, vascular endothelial cells and macrophages. Because the organoid culture system allows expansion of Lgr5-positive stem cells and other epithelial cells derived from stem cells, it is possible to observe the biological signatures of the tumor cells alone, to the exclusion of non-epithelial cells. This may explain the discrepancy in gene expression between tumor organoids and tumor tissues.

Our results demonstrated that induction of miR-194 suppressed the growth of tumor organoids by repressing expression of Mdm2 and E2f3. In contrast, induction of miR-215 did not suppress the growth of tumor organoids. Ctnnbip1 is a negative regulator of the Wnt/β-catenin signaling pathway. Induction of miR-215 suppressed Ctnnbip1 as its target gene, suggesting that it may promote the proliferation of tumor organoids via activation of the Wnt/β-catenin signaling pathway. Stem cell markers such as Lgr5 and Ereg were suppressed in tumor organoids after induction of miR-215, although Lgr5 is not a direct target of miR-215. We speculate that in tumor organoids transfected with miR-215, Lgr5 expression is suppressed due to loss of stemness.

Induction of miR-215 reduced the number of organoids, whereas the size of the organoids was increased. The finding that miR-215 decreased the number of tumor organoids recapitulates a recent study showing that the number of colonies formed by colon cancer cell lines overexpressing miR-215 was decreased (21). In addition, miR-215-transfected tumor organoids showed significantly increased sensitivity to irinotecan, suggesting that miR-215 inhibits the stemness of tumor organoids. These results are consistent with the report described above in which miR-215 inhibited the stemness of colon cancer cells in 3D culture (21). Thus, miR-215 is considered to suppress the stemness of cancer cells and tumor organoids. However, as shown in Figure 2(f), miR-215 induced the formation of larger tumor organoids, suggesting that the cells in large organoids transfected with miR-215 are more differentiated than those in small organoids.

To examine the expression levels of miRNA in stem cells, we performed flow cytometry to sort Lgr5-positive stem cells in tumor organoids, as shown in Figure S1(b). We then examined the expression levels of miR-192, miR-194 and miR-215 in both Lgr5-positive stem cells and Lgr5-negative cells by quantitative RT-PCR. As shown in Figure S1(b), no expression of miR-194 and miR-215 was detected in Lgr5-positive stem cells, suggesting that miR-194 and miR-215 were expressed in differentiated cells, but not in Lgr5-positive stem cells. These findings are consistent with our observation that miR-215 suppressed the stem cell signatures, as shown in Figure 4.

To confirm the tumorigenesis of organoids derived from tumors of Apcmin/+ mice, we implanted tumor organoids (1.0 × 10^5 cells) subcutaneously into the backs of SCID mice. However, at approximately 2 months after implantation, no tumors had formed (data not shown). Because tumors of Apcmin/+ mice are adenomas, and, thus, at an early stage of tumorigenesis, they do not have the ability to form tumors in SCID mice. In order to produce in vivo models of tumorigenesis, additional driver gene mutations such as Kras, Tp53 and Smad4 in the tumor organoids might be necessary. Further experiments such as introduction of driver gene mutations in tumor organoids using the CRISPR/Cas9 system will be necessary.

Taken together, these results indicate that miR-194 is relevant to growth suppression, whereas miR-215 inhibits the stemness of tumor organoids. miR-215 alone did not affect the growth of organoids, but miR-194 and 215 showed synergetic effects on apoptosis and growth suppression of tumor organoids. In the future, replacement of miR-194 and miR-215 may lead to the prevention and treatment of colorectal cancer. The concept of this therapy is to restore expression of miR-194 and miR-215 in colorectal cancers to a level comparable to that in surrounding non-cancer tissues. Further studies to investigate tissue-specific delivery of miRNA and potential off-target side effects will be necessary to develop an effective miRNA-based cancer therapy.

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Disclosure Statement
The authors have no conflicts of interest to declare.
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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Flow cytometry analysis of Lgr5 in intestinal tumor organoids. (a) Flow cytometry analysis of Lgr5 expression in intestinal tumor organoids transfected with miR-194, miR-215 and the cluster. (b) Flow cytometry analysis for sorting Lgr5-positive stem cells in intestinal tumor organoids (upper). Quantitative RT-PCR analysis of miR-192, 194 and 215 normalized to U6 in Lgr5-positive and negative cells (lower).

**Table S1.** Primer sequences used for constructing vectors encoding miRNA

**Table S2.** Primer sequences used for quantitative RT-PCR

**Table S3.** Clinicopathological features of the colorectal cancer patients, including age, sex, tumor location, tumor size, histologic type and TNM stage.