

miR-137 Regulates the Tumorigenicity of Colon Cancer Stem Cells through the Inhibition of DCLK1

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Abstract

miRNAs have important roles in regulating cancer stem cell (CSC) properties and are considered to be potential therapeutic targets. However, few studies have focused on miRNAs which are specifically related to colon CSCs. Here, a PCR-based miRNA profiling analysis of normal colon stem cells (NCSC) and colon CSCs (EpCAM⁺/CD44⁺/CD66a⁻) identified miRNAs which regulate colon CSC properties. Interestingly, miRNA-137 (miR-137) expression was downregulated in the colon CSCs compared with NCSCs, while *doublecortin-like kinase 1* (*DCLK1*) mRNA was highly expressed in the colon CSCs but low in the NCSCs. In fact, *DCLK1*-positive cancer cells were widely distributed in clinically resected colon cancer specimens, while *DCLK1*-positive epithelial cells were rarely detected in normal colon tissues including the crypt bottoms. Luciferase assay and immunoblot analysis revealed that miR-137 regulated *DCLK1* gene

expression. Transduction of exogenous miR-137 suppressed the development of colon cancer organoids *in vitro* and the tumorigenicity of colon cancer cells *in vivo* without affecting the growth of normal intestinal organoids. Furthermore, the suppression of miR-137 enhanced the organoid development of normal colon cells. These data demonstrate that miR-137 has the capacity to suppress the tumorigenicity of colon CSCs and that maintained expression of miR-137 in NCSCs contributes to suppressing uncontrolled cell proliferation through the inhibition of *DCLK1* expression.

Implications: The miR-137/*DCLK1* axis as an important regulator in NCSCs and colon CSCs; further understanding of this axis may foster the development of potential gene therapeutic strategies targeting colon CSCs. *Mol Cancer Res*; 1–9. ©2016 AACR.

Introduction

Stem cells (SC), which possess self-renewal and differentiation abilities, are responsible for the organization of normal tissues and organs. Much research in recent years suggests that cancer tissues arise from a small subpopulation of long-lived tumor cells termed cancer stem cells (CSC; ref. 1). CSCs are potential therapeutic targets because they are considered to be responsible for therapeutic tolerance and recurrence, which lead to poor outcome in cancer patients (2–4). There is growing concern about the mechanisms that regulate the properties of CSCs.

Recent studies show that several miRNAs, which post-transcriptionally regulate the expression of certain genes, regulate the properties of CSCs (5). A miRNA is a small non-coding RNA that binds to the sequence within the 3' untranslated region (3'UTR) of its target mRNA and functions as a guide in RNA silencing. The 2–8

nucleotides from the 5' end of miRNA, known as the miRNA seed sequence, play an indispensable role in its recognition of its target mRNA. More than 60% of human protein-coding genes contain at least one preserved site, which matches the miRNA seed sequence (6–8). Each miRNA is able to control the expression of hundreds of target mRNAs simultaneously and to regulate various biologic functions, including cellular proliferation, differentiation, and apoptosis (9). miRNAs are also associated with cancers and they can function as tumor suppressors or oncogenes. miRNAs have therefore been regarded as attractive targets in the development of more powerful therapies. In fact, several studies have reported miRNA therapies, including anti-miRNA and miRNA replacement, are in the preclinical and clinical stage of development for cancer treatments for breast and prostate cancers (10). However, miRNA-based therapies that specifically target colon CSCs have yet to be developed.

The SC population that contains colon CSC properties is identified by its surface markers, namely CD133, CD44, LGR5, and ALDH1 (11–16). Normal colon stem cells (NCSC) share most of these markers, which limit their application as the therapeutic targets. Recently, Nakanishi and colleagues demonstrated that doublecortin-like kinase 1 (*DCLK1*) is a surface marker that is unique to mouse adenoma tumor stem cells. The elimination of *DCLK1*-positive cells resulted in the significant regression of polyps without affecting the normal intestine in a mouse model (17). Therefore, *DCLK1* could be a distinctive marker for human colon CSCs.

In this study, we present that the miRNA-137/*DCLK1* axis plays an important role in the formation of cancer tissues. We found that miRNA-137 (miR-137) was downregulated and that

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DCLK1 was highly expressed in colon CSCs, but miR-137 was upregulated and DCLK1 was low expressed in NCSCs, and that miR-137 regulated directly the expression of DCLK1. miR-137-transduced colon cancer cells were observed to suppress organoid development and to repress tumorigenicity *in vivo*. Our findings may help to develop a novel miRNA-based therapeutic strategy which targets colon CSCs without affecting normal colon tissues.

Materials and Methods

Cell line and cell culture

SW480 human colon cancer cell line was purchased from ATCC. SW480 cells were cultured in DMEM (Nacalai Tesque) containing 10% FBS (Life Technologies) and penicillin (100 U/mL) and streptomycin (100 µg/mL; Life technologies), and were used in the early passages in all of the experiments. SW480 cell authenticity was evaluated by utilizing Short Tandem Repeat profiling. Human primary colon cells (hPCC, T4056) were purchased from Applied Biological Materials Inc. (abm) and cultured in plates pretreated with Applied Cell Extracellular Matrix (abm) in DMEM with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL; Life technologies). HPCCs were used in less than 7 passages in all of the experiments for fewer than 6 months after receipt.

RNA isolation and quantitative reverse-transcriptase PCR

RT, pre-PCR, and the real-time PCR for miRNA expression profiles were performed by the real-time PCR method as previously described (18, 19). Total RNA was isolated using a High Pure miRNA isolation Kit (Roche), according to the manufacturer's instructions. A SuperScriptIII First-Strand Synthesis System for RT-PCR (Life Technologies) and a TaqMan MicroRNA Reverse Transcription kit (Life Technologies) were used for the reverse transcription of mRNA and miRNA, respectively. The RT products were amplified with TaqMan PreAmp master Mix (Life Technologies). The abundance of each mRNA or miRNA was measured using a 7900HT Fast Real-Time PCR system (Applied Biosystems). All of the miRNA data were normalized by the amount of U6snRNA, while all of the mRNA data were normalized by the amount of *ACTB*.

Preparation of single-cell suspensions

Primary colon cancer specimens and normal colon specimens were obtained from the consented patients who underwent colectomy as approved by the Research Ethics Boards at Kyoto University Hospital (Kyoto, Japan). Human colon specimens and mouse xenograft tumors were enzymatically dissociated into a single-cell suspensions using a gentleMACS Dissociator and Human Tumor Dissociation Kit (Miltenyi Biotec), according to the manufacturer's protocols.

Flow cytometry

The single-cell suspensions from the human colon specimens, mouse xenograft tumors, and cultured cells were immunohistologically stained with a mouse anti-human EpCAM antibody (Clone: EBA-1, Becton, Dickinson and Company; BD), a mouse anti-human CD44 antibody (Clone: G44-26, BD), and a mouse anti-human CD66a antibody (Clone: 283340, R&D Systems Inc.). After washing, the cells were resuspended with Hank's Balanced Salt Solution (Sigma-

Aldrich) containing 2% FBS, and dead cells were labeled with 0.5 µg/mL 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Life Technologies). These samples were analyzed using a FACS Aria II cell sorter (BD).

Plasmid vectors for luciferase reporter assays and mutagenesis

A 170-bp fragment of the *DCLK1* 3'UTR (corresponding to positions of 3364-3533 of the NM_00195415) and a 165-bp fragment of the *DCLK1* 3'UTR (corresponding to positions of 5791-5955 of the NM_00195415) were amplified by a PCR using the cDNA of HEK293T cells as a template, and cloned into the pGEM-T vector (Promega, LLC.). Each *DCLK1* 3'UTR product was then cloned at the 3' of the luciferase gene of pGL3-MC vector (19). The mutation of the two putative miR-137 target sequences within the 3'UTR of *DCLK1* was generated using a QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). All products were sequenced.

Lentivirus backbone plasmid

The miR-137 sequence, including the stem loop structure and 200–300 bp of the upstream and downstream flanking genomic sequence, was cloned by PCR using the genomic DNA of HEK293 cells as a template. The products were cloned into multicloning sites of pEIZ-HIV-ZsGreen vector (19). In the same manner, the *DCLK1* sequence (NM_00195415.1), which does not contain the 3'UTR sequence, was cloned by PCR using the mRNA of SW480 cells as a template. The products were cloned into multicloning sites of the pEIZ-HIV-dTomato vector. All products were sequenced. The anti-miR-137-miRNA construct (MZIP-137-PA-1) and the scrambled control RNA-expressing plasmid were purchased from System Biosciences. The pGIPZ lentiviral vectors for DCLK1 (V2LHS_36415, V2LHS_36418, V3LHS_395785) and the GIPZ nonsilencing lentiviral shRNA vector were purchased from GE Dharmacon. Lentiviruses were produced as previously described (20).

Luciferase reporter assay

SW480 cells were seeded at 1×10^5 cells per well in a 48-well plate the day before transfection. All transfections were carried out using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Cells were transfected with 320 ng of pGL3 luciferase expression construct containing the 3'UTR of human *DCLK1*, 16 ng of pRL-TK *Renilla* luciferase vector (Promega), and 25 nmol/L of negative control or hsa-miR-137 precursor (Life Technologies). Forty-eight hours after transfection, the luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and normalized to *Renilla* luciferase activity. All experiments were performed in triplicate.

Western blotting

SW480 cells were transfected by 25 nmol/L of negative control or hsa-miR-137 precursor and cultured for 2 days. The cells were lysed with Nonidet-P40 (NP-40) buffer (150 mmol/L sodium chloride, 1.0% NP-40, 50 mmol/L Tris, pH 8.0). The samples were subjected to SDS-10% PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk, an immunoblotting with a rabbit anti-human

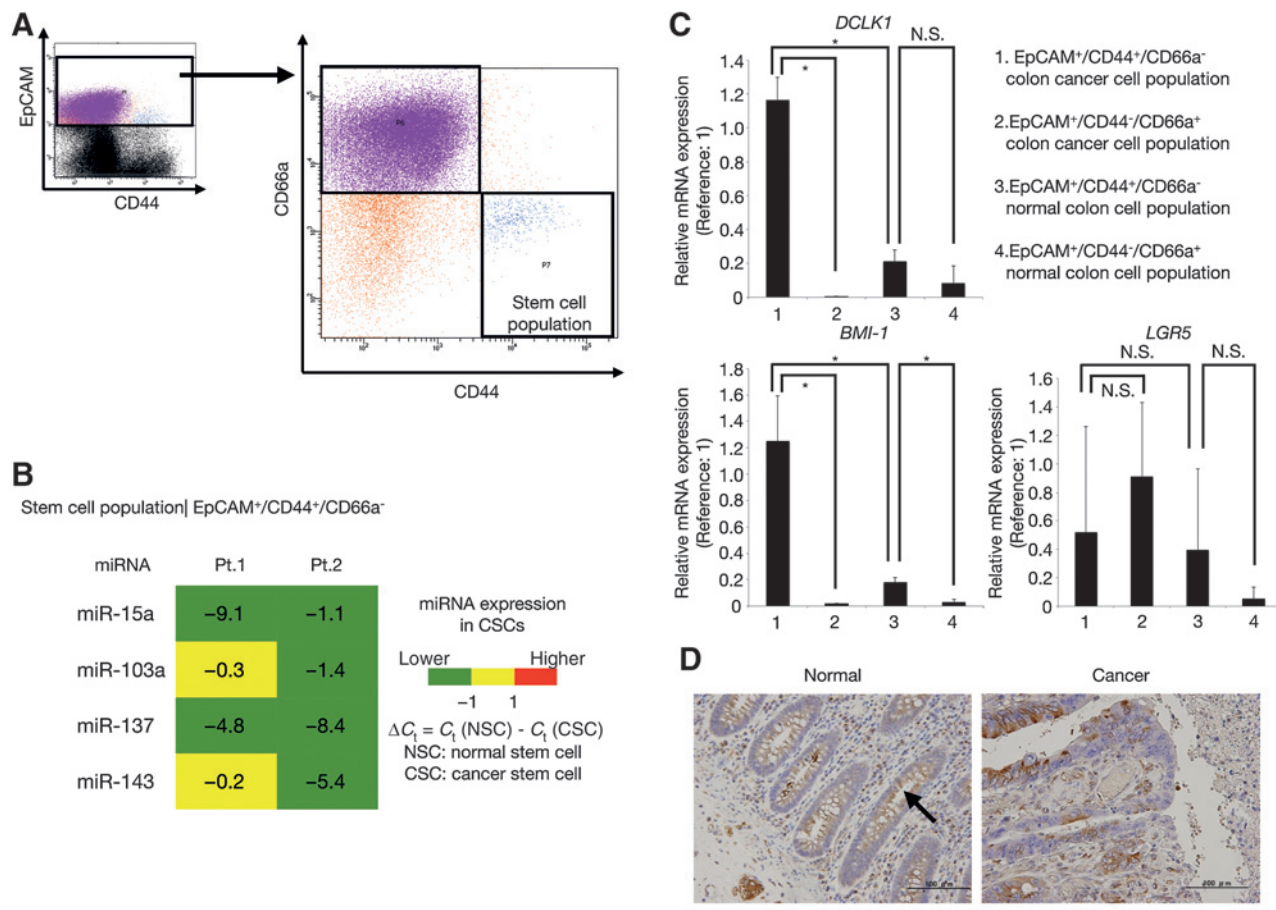


Figure 1.

Analysis of human normal colon and colon cancer specimens. A, representative flow-cytometric plot. EpCAM⁺/CD44⁺/CD66a⁻ cells and EpCAM⁺/CD44⁺/CD66a⁺ cells in both colon cancer tissues and normal colon tissues were collected by flow cytometry. B, expression pattern of 384 miRNAs in the EpCAM⁺/CD44⁺/CD66a⁻ population of colon cancer tissues. The amount of miRNA expression (C_t value) was analyzed by multiplex quantitative real-time PCR. Only miR-15a and miR-137 were suppressed in the EpCAM⁺/CD44⁺/CD66a⁻ population of both colon cancer specimens. Numbers indicate the difference of C_t values (ΔC_t) between normal stem cells and CSCs. C, qRT-PCR results of several intestinal stem cell markers. The *DCLK1* mRNA expression in the EpCAM⁺/CD44⁺/CD66a⁻ colon cancer cell population was significantly higher than that of EpCAM⁺/CD44⁺/CD66a⁺ colon cancer cell population and EpCAM⁺/CD44⁺/CD66a⁻ normal colon cells ($n = 3$, *, $P < 0.05$, N.S., not significant). D, IHC analysis. Normal tissues adjacent to cancers showed positive DCLK1 immunoreactivity but DCLK1-positive cells were rare (allow) and no cells located at the bottom of the crypts (left). Cancer tissues showed diffuse DCLK1-positive pattern (right). Scale bar: 100 μ m.

DCLK1 antibody (Clone: EPR6085, abcam), followed by a horseradish peroxidase-conjugated secondary antibody, was performed. A LASO-3000 mini system (Fuji Film) was used to determine chemiluminescence.

Organoid growth assay

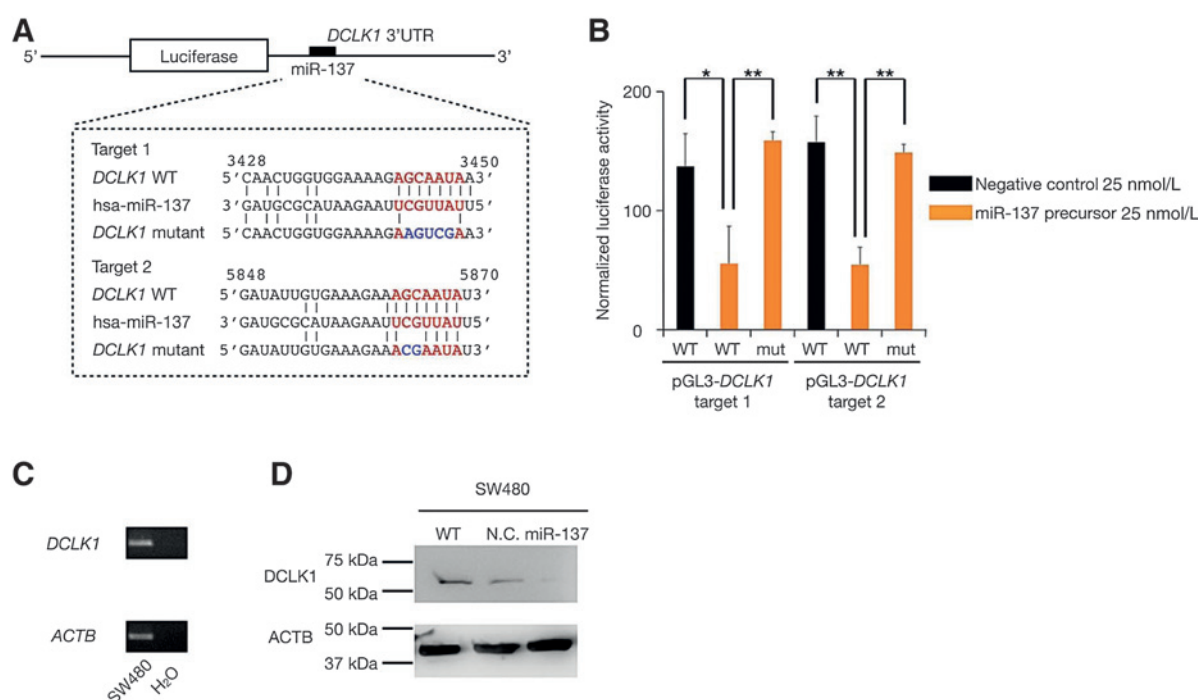
SW480 cells were coinfectd with the miR-137-GFP-expressing lentivirus or the GFP-control lentivirus, and the DCLK1-mCherry expressing lentivirus or the mCherry-control lentivirus and cultured for 7 days. The GFP⁺/mCherry⁺/CD44⁺ SW480 cells were collected by flow cytometry and seeded at 2,000 cells per well in a 96-well plate. The numbers of organoids more than 100 μ m were counted 10 days after seeding.

The mouse small intestinal crypts were isolated and cultured as described (21). The organoids were cultured in a 96-well plate with standard ENR medium [50 ng/mL of murine recombinant EGF (Life Technologies), 100 ng/mL of murine recombinant Noggin (PeproTech) and 500 ng/mL of human recom-

binant R-spondin1 (R&D)] for 7 days. Lentivirus was produced in a 100-mm dish and concentrated. Briefly, the collected viral suspension was passed through a 0.45- μ m filter and centrifuged at 2,700 $\times g$ for 30 minutes at 4°C. After discarding the supernatant, the pellets were centrifuged at 2,700 $\times g$ for 5 minutes at 4°C. After discarding the remaining supernatant, the pellets were suspended in 100 μ L of OPTI-MEM1 (Life technologies; ref. 22). Organoids were removed from the Matrigel and mechanically dissociated. Dissociated organoids were infected with 100 μ L of lentiviral suspension by spinoculation (23). The infected organoids were transferred to fresh Matrigel with a 1:4 split ratio. All experiments were performed in triplicate.

Immunohistological staining

Formalin-fixed, paraffin-embedded sections derived from the human colon cancer tissues were stained with anti-human DCLK1 rabbit monoclonal antibody (Clone: EPR6085, abcam) by the

**Figure 2.**

Targeting *DCLK1* by miR-137. A, schematic representation of the predicted miR-137 target site sequence within the 3'UTR of *DCLK1*. *DCLK1* had two predicted target sites (target 1 and target 2). Five nucleotides within target 1 and two nucleotides within target 2 complementary to the seed sequence (the nucleotide 2-7 of miRNA) of miR-137 were mutated in the *DCLK1*-mutant plasmid. The number indicates the position of the nucleotides in the wild-type sequence of *DCLK1* (NM_00195415). B, activity of luciferase gene linked to the 3'UTR of *DCLK1*. The pGL3 luciferase reporter plasmids with the wild-type (WT) or mutated 3'UTR of *DCLK1* (mut) were transiently infected into SW480 cells along with 25 nmol/L negative control or miR-137 precursor. Cotransfected *Renilla* luciferase reporter was used for the normalization. Luciferase activities were measured after 48 hours. The data are mean and SD of separated transfections ($n = 3$, *, $P < 0.05$, **, $P < 0.01$). C, semiquantitative RT-PCR analysis of *DCLK1* mRNA expressions of colon cancer cell line SW480. SW480 cells expressed *DCLK1*. D, suppression of endogenous *DCLK1* protein. SW480 cells were transfected with 25 nmol/L negative control (N.C.) or miR-137 precursor, and *DCLK1* protein level was analyzed by Western blotting after 48 hours.

avidin–biotin immunoperoxidase method. Microwave antigen retrieval was performed.

Proliferation assay

The SW480 cells were coinfecting with the miR-137-GFP-expressing lentivirus and the DCLK1-mCherry expressing lentivirus or the control lentivirus and cultured for 7 days. The GFP⁺/mCherry⁺/CD44⁺ SW480 cells were collected by flow cytometry and seeded at 5,000 cells per well in a 48-well plate. The cells were counted on Days 1, 3, 5, 7, and 10. All experiments were performed in triplicate.

In vivo tumorigenicity assay

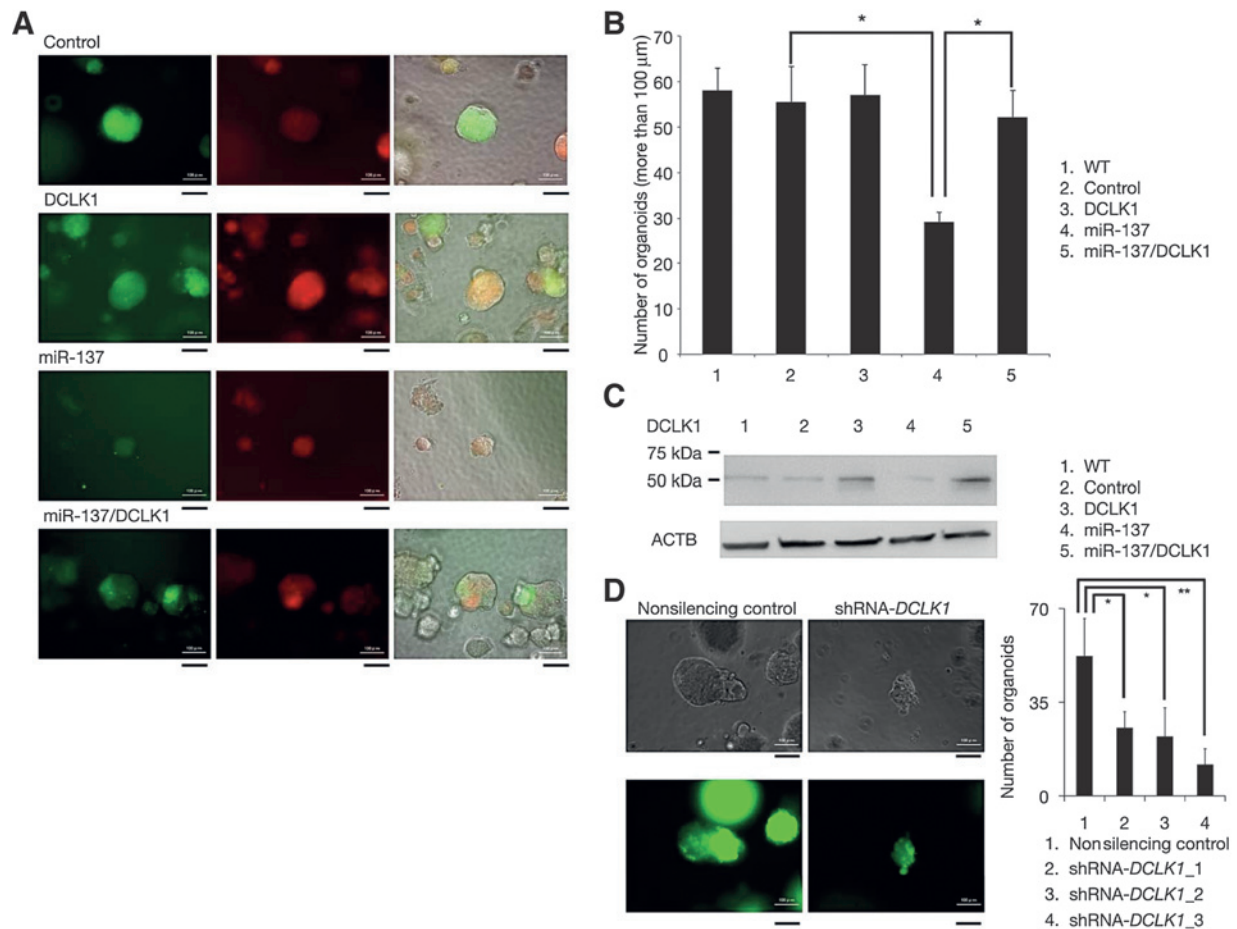
SW480 cells were coinfecting with the miR-137-GFP-expressing lentivirus or the GFP-control lentivirus, and the DCLK1-mCherry expressing lentivirus or the mCherry-control lentivirus and cultured for 7 days. The GFP⁺/mCherry⁺/CD44⁺ SW480 cells were collected by flow cytometry and cultured for another 7 days. A total of 2×10^6 cells in 100 μ L of serum-free PBS were injected subcutaneously into both dorsal flanks of immunodeficient nude mice (KSN/Slc mouse, SLC) and non-obese diabetic/severe combined immunodeficient mice (NOD.CB17-Prkdc^{scid}/J, OBS). The volume was calculated by the formula $0.5 \times L \times W^2$ (L: Length, W: Width). The experiments

were reviewed and approved by the Animal Ethics and Research Committee, Kyoto University (Permit number: 13553, 14209, 15188), and were conducted in accordance with institutional guidelines. All efforts were made to minimize the suffering of the animals.

Results

The miRNA and DCLK1 profiling of human normal colon and colon cancer specimens

First, we collected the EpCAM⁺/CD44⁺/CD66a[−] population in which NCSCs or tumorigenic colon CSCs were concentrated and the EpCAM⁺/CD44[−]/CD66a⁺ population in which differentiated cells were concentrated by using flow cytometry (Fig. 1A; ref. 24). We then evaluated the relative amounts of 384 miRNAs in the EpCAM⁺/CD44⁺/CD66a[−] population in the normal colon and colon cancer tissues isolated from two human colon cancer specimens using a multiplex real-time PCR. Among 384 miRNAs, the expression of only miR-15a and miR-137 was lower in the EpCAM⁺/CD44⁺/CD66a[−] population of colon cancer cells than that of normal colon cells (Fig. 1B). Next, we analyzed the *DCLK1* mRNA expression in the each population of normal colon and colon cancer tissues using a real-time PCR. The EpCAM⁺/CD44⁺/CD66a[−] colon cancer cell population showed the significantly increased mRNA expression of *DCLK1* in comparison to the

**Figure 3.**

Suppression of organoid development by miR-137 through inhibiting the expression of *DCLK1* in SW480 cells. A, representative images of the organoids derived from GFP⁺/mCherry⁺/CD44⁺ SW480 cells. The left and middle panels are the fluorescent microscopic images for the detection of GFP and mCherry, respectively. The right panels are the phase-contrast images of the organoids merged with the images of the green fluorescent images (left) and the red fluorescent images (middle). Scale bar: 100 μm. B, MiR-137 suppressed colon cancer organoid development. The data are mean ± SD (n = 3, *, P < 0.01). C, the *DCLK1* expression was suppressed in the organoids derived from SW480 cells transfected with miR-137 expressing lentivirus. D, representative images of organoids of SW480 cells transfected with the pGIPZ lentiviral non-silencing control or the pGIPZ lentiviral shRNA against *DCLK1* (shRNA-*DCLK1*). The organoid development with the shRNA-*DCLK1* was suppressed compared with that with the non-silencing control. The top panels are the phase-contrast images of the organoids, and the bottom panels are the fluorescent microscopic images for the detection of GFP. Scale bar: 100 μm. The data are mean ± SD (n = 4, *, P < 0.05, **, P < 0.01).

EpCAM⁺/CD44⁺/CD66a⁺ colon cancer cell population and the EpCAM⁺/CD44⁺/CD66a⁻ normal colon cell population. Furthermore, we found no difference in the *DCLK1* expression between both populations in normal colon tissues. We also analyzed the mRNA expressions of the two intestinal stem cell markers, *BMI-1* and *LGR5*. The mRNA expression of *BMI-1* in the EpCAM⁺/CD44⁺/CD66a⁻ population in both tissues was significantly higher than that in the EpCAM⁺/CD44⁺/CD66a⁺ population. The mRNA expression of *LGR5* was not significantly increased in the EpCAM⁺/CD44⁺/CD66a⁻ colon cancer cell population (Fig. 1C). IHC analyses (Fig. 1D and Supplementary Fig. S1A) showed that normal colon tissues adjacent to cancers were rarely positive for *DCLK1* and the positive cells were not located at the bottom of the crypts where NCSCs exist (25). In contrast, the human colon cancer tissues were widely positive for *DCLK1*.

MiR-137 targets *DCLK1*

According to TargetScanHuman 6.2 (<http://www.targetscan.org/>; ref. 26), *DCLK1* was a potential target of miR-137 but it was not a target of miR-15a. To assess the ability of miR-137 to regulate the expression of *DCLK1* at the mRNA level, we performed luciferase reporter assays. The two predicted target sites for miR-137 were located within the 3'UTR of the *DCLK1* mRNA (we named them target 1 and target 2; Fig. 2A). We therefore cloned each target site into the pGL3-Control vector, downstream of a luciferase minigene. Colon cancer cell line SW480 cells were cotransfected with the pGL3-Control vector, the pRL-TK *Renilla* luciferase vector, and the negative control or miR-137 precursor. We found that the cotransfection of the miR-137 precursor suppressed the luciferase activity of the vectors, which had target 1 or target 2 (Fig. 2B). Furthermore, mutations within the predicted target sites abolished the

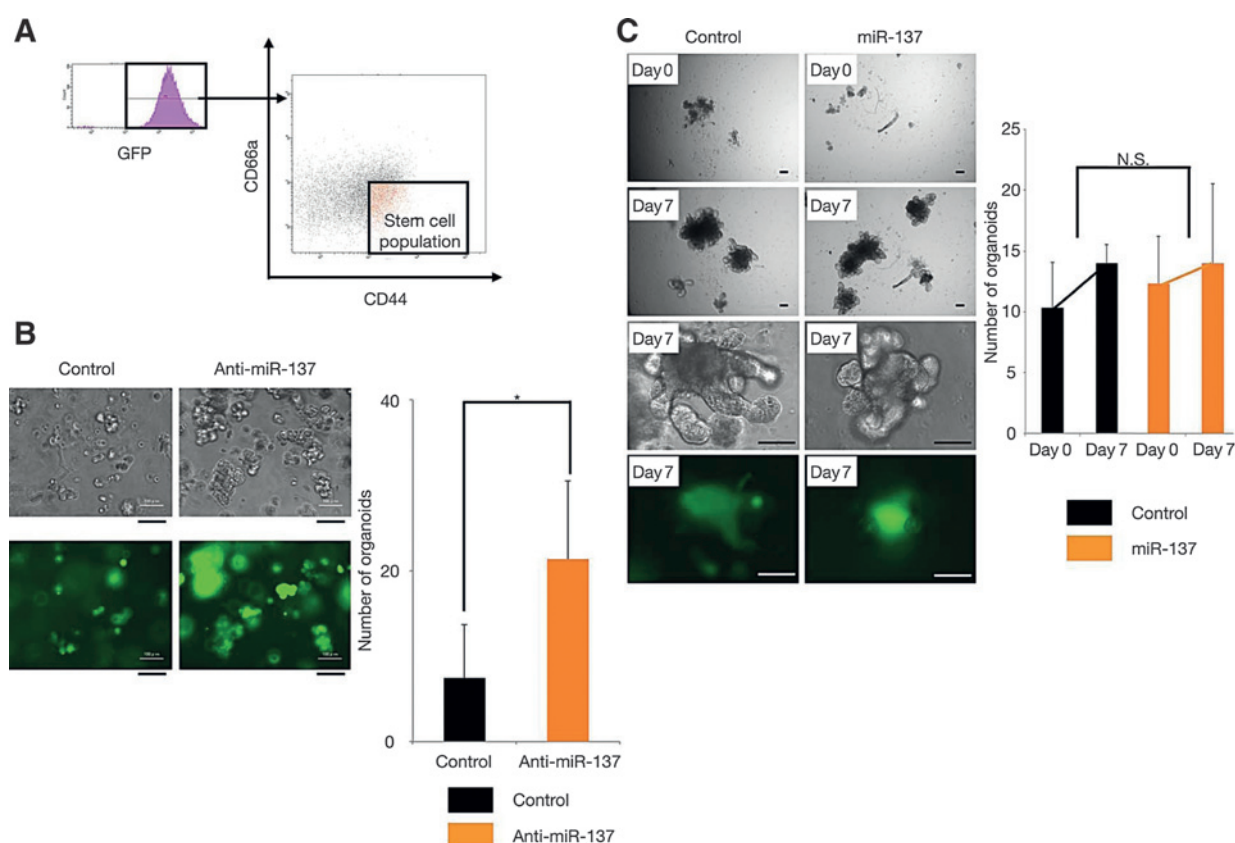


Figure 4. Influence of miR-137 on normal intestinal organoid. A, hPCCs infected by control lentivirus or anti-miR-137-expressing lentivirus were cultured for 7 days. GFP⁺/CD44⁺/CD66a⁻ hPCCs were collected by flow cytometry. B, Anti-miR-137 enhanced organoid development. Representative images of the organoids derived from GFP⁺/CD44⁺/CD66a⁻ hPCCs. The top panels are the phase-contrast images of the organoids, and the bottom panels are the fluorescent microscopic images for the detection of GFP. Scale bar: 100 μ m. The data are mean $\pm$ SD ($n = 9$, $P < 0.01$). C, organoids derived from mouse small intestine infected with miR-137-expressing lentivirus grew as well as those infected with control lentivirus. The bottom panels are the fluorescent microscopic images for the detection of GFP, and the other panels are the phase-contrast images of the organoids. Day0 and Day7 indicate the day of infection and 7 days after infection, respectively. Scale bar: 100 μ m. The data are mean $\pm$ SD ($n = 6$, N.S., not significant, $P > 0.05$).

inhibitory ability of miR-137 (Fig. 2B). We then evaluated the ability of miR-137 to regulate the DCLK1. We confirmed that SW480 cells expressed *DCLK1* mRNA by RT-PCR (Fig. 2C), and found that the protein level of DCLK1 was decreased in the SW480 cells (Fig. 2D).

MiR-137 suppresses the development of colon cancer organoids through the inhibition of *DCLK1* expression

The organoid culture system which recapitulates the crypt-villus architecture of the intestinal epithelium and retains the cellular hierarchy of the intestinal tissue has been widely used (21). We tested the ability of miR-137 to regulate the organoid development of the colon cancer cell line SW480 and murine normal intestinal cells. SW480 cells were infected with the miR-137-GFP expressing lentivirus or the GFP-control lentivirus, and the DCLK1-mCherry expressing lentivirus or the mCherry-control lentivirus, and cultured for 7 days. The GFP⁺/mCherry⁺/EpCAM⁺/CD44⁺/CD66a⁻ SW480 cells were then collected by flow cytometry (Supplementary Fig. S2A and S2B). The sorted 2,000 cells were embedded in Matrigel and cultured for 10 days. The number of organoids infected with the miR-137 expressing lentivirus was significantly lower than that of the organoids

infected with the control lentivirus (Fig. 3A and 3B). We confirmed that DCLK1 expression was suppressed in the organoids derived from SW480 cells transfected with miR-137 expressing lentivirus (Fig. 3C). To confirm the functional relevance of DCLK1 regulation by miR-137, we constructed a DCLK1 expressing lentivirus in which the *DCLK1* cDNA does not contain the 3'UTR sequence. Coexpression of this *DCLK1* transgene substantially rescued the defect in organoid formation of SW480 cells infected with the miR-137-expressing lentivirus (Fig. 3B and C). Next, to determine whether DCLK1 regulated the organoid development of colon cancer cells, SW480 cells were transduced with the pGIPZ lentiviral non-silencing control shRNA (Control-SW480) or the pGIPZ lentiviral shRNAs against *DCLK1* which target sequences in the 3'UTR of *DCLK1* (shDCLK1-SW480). We confirmed that DCLK1 expression was suppressed in the shDCLK1-SW480 cells (Supplementary Fig. S2C). The number of organoids derived from the shDCLK1-SW480 cells was significantly lower than that of organoids from the Control-SW480 cells (Fig. 3D). We also found that coexpression of the exogenous DCLK1 substantially rescued the defect in organoid formation of shDCLK1-SW480 cells (Supplementary Fig. S2D).

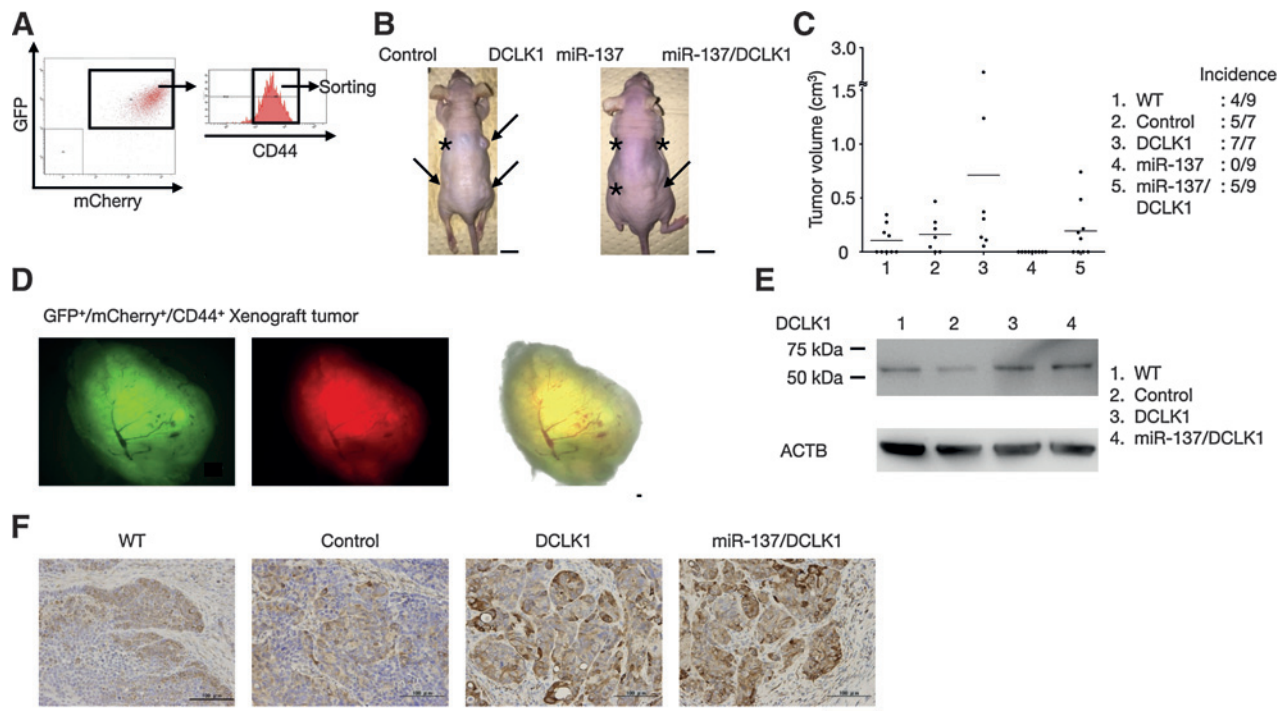


Figure 5.

Suppression of tumorigenicity of SW480 cells by miR-137. A, a representative flow cytometry plot. GFP⁺/mCherry⁺/CD44⁺ SW480 cells were collected by flow cytometry. B, a representative image of the tumors in a mouse injected with GFP⁺/mCherry⁺/CD44⁺ SW480 cells. After sorting by flow cytometry, 2.0×10^6 cells were injected subcutaneously into nude mice. Scale bar: 10 mm (C): The tumor volume and incidence were measured 6 weeks after injection. MiR-137 suppressed tumor formation of SW480 cells. ($n = 9$ in three independent experiments, except control and DCLK1, in which $n = 7$; D): A representative image of the xenograft tumor derived from GFP⁺/mCherry⁺/CD44⁺ SW480 cells. The left and middle panels are the fluorescent microscopic images for the detection of GFP and mCherry, respectively. The right panel is the phase-contrast image of the tumor merged with the images of the green fluorescent images (left) and the red fluorescent images (middle). Scale bar: 100 μ m. E, DCLK1 expression of the xenograft tumors derived from GFP⁺/mCherry⁺/CD44⁺ SW480 cells. F, formalin-fixed, paraffin-embedded sections derived from the xenograft tumors were stained with anti-human DCLK1 rabbit monoclonal antibody by the avidin-biotin immunoperoxidase method. Scale bar: 100 μ m.

MiR-137 knockdown enhances organoid development derived from human primary colon cells

To evaluate the ability of miR-137 to regulate the NCSCs, human primary colon cells (hPCCs) were infected with the control or anti-miR-137-GFP-expressing lentivirus and cultured for 7 days. The GFP⁺/EpCAM⁺/CD44⁺/CD66a⁻ cells were collected by flow cytometry (Supplementary Fig. S3A; Fig. 4A) and the 4,000 cells were embedded in Matrigel with ENR medium. The number of organoid larger than 100 μ m was counted 7 days later. The number of organoids infected with the anti-miR-137-expressing lentivirus was significantly higher compared with that of organoids infected with control lentivirus (Fig. 4B). We confirmed that DCLK1 expression was enhanced by the knockdown of miR-137 (Fig. S3B). Next, we infected organoids derived from the mouse small intestine with the control lentivirus or the miR-137-expressing lentivirus. According to TargetScanHuman 6.2, murine *Dclk1* has one target site of miR-137 which is in common with human target 1 (Supplementary Fig. S3C). We observed that the rate of increase of the organoids infected with the miR-137-expressing lentivirus was similar to that of the organoids infected with the control lentivirus (Fig. 4C).

MiR-137 suppresses tumorigenicity of colon cancer cells

To assess whether miR-137 suppressed the tumorigenicity of colon cancer cells, we subcutaneously injected the GFP⁺/

mCherry⁺/CD44⁺ SW480 cells into immunodeficient mice (Fig. 5A), and then we measured the incidence and the volume of tumors at 6 weeks after injection. We observed that a lower incidence and smaller tumors in mice injected with the cells infected by the miR-137-expressing lentivirus in comparison with the mice injected with the cells infected by the control lentivirus and that coexpression of the DCLK1 transgene substantially rescued the defect in tumor formation of SW480 cells infected with the miR-137-expressing lentivirus (Fig. 5B and C; and Supplementary Fig. S4A). The tumors were composed of GFP-positive and mCherry-positive cells (Fig. 5D). We confirmed the DCLK1 expression of the tissues formed by the GFP⁺/mCherry⁺/CD44⁺ SW480 cells (Fig. 5E and F). We found that miR-137 did not affect the proliferation of SW480 cells (Supplementary Fig. S4B). Taken together, these data indicate that miR-137 suppresses the tumorigenicity of the colon CSCs through the inhibition of *DCLK1* and that miR-137 does not affect the NCSCs.

Discussion

We showed that the mechanism in which miR-137 regulates the expression of *DCLK1*, and demonstrated the opposite expression patterns of miR-137/DCLK1 in human NCSC and colon CSC. It is therefore speculated that the dysfunction of the miR-137/DCLK1

axis in colon CSCs would play an important role in tumorigenicity of colon CSCs.

We collected EpCAM⁺/CD44⁺/CD66a⁻ cells as a SC population and EpCAM⁺/CD44⁺/CD66a⁺ cells as a differentiated cell population from both human normal colon and colon cancer tissues by using flow cytometry as previously described (24). Although the EpCAM⁺/CD44⁺/CD66a⁻ SC population was rather heterogeneous, the DCLK1 expression was significantly higher in the colon CSC population as compared with the differentiated population, while the expression level was not significantly different between NCSCs and the differentiated cells of the normal colon. These data suggested that DCLK1 is a distinct colon CSC marker which is not shared with NCSCs.

In this study, DCLK1-positive epithelial cells were found in normal colon tissues but they were not located at the crypt bottoms, what we call intestinal stem cell niches. Our findings in the normal colon tissues are compatible with previous reports that implicated DCLK1 as a differentiation marker in normal tissue (27, 28). In contrast, DCLK1-positive cancer cells were widely distributed not only in the primary colon cancer region, but also metastatic regional lymph nodes (Supplementary Fig. S1B). Interestingly, the DCLK1-positive cancer cells were dominant in the metastatic regional lymph nodes. This finding suggests that DCLK1 is related to colon cancer metastasis.

The profile of the EpCAM⁺/CD44⁺/CD66a⁻ population in normal colon tissues and colon cancer tissues indicated that the expression of miR-137 was inversely correlated with that of DCLK1. We conducted a luciferase reporter assay to test the ability of miR-137 to regulate the 3'UTR of *DCLK1*. Although we did not assess the luciferase activity of the pGL3-Control vector which had both target sequences, our experimental fidelity was maintained because the activity of the each vector which has each site was significantly suppressed by miR-137. The results of the luciferase reporter assay and Western blotting analysis suggest that miR-137 targets the *DCLK1* mRNA and suppresses the protein level of DCLK1.

The organoid culture system is an established culture system for single stem cells without a mesenchymal niche, which is able to develop the organoids that represent the characteristics of the original epithelial cells (21, 29). In addition, intestinal tumor organoids also recapitulate intestinal tumorigenesis (30). We applied this system to evaluate the function of miR-137 in a human cancer cell line, hPCCs and the murine small intestine. Murine *Dclk1* had one predicted target site of miR-137 and the sequence was the same as that of human target 1. This finding confirmed the high fidelity of our study using the murine small intestine. The results of organoid growth assays indicate that miR-137 specifically suppresses the development of organoids derived from colon CSCs through the inhibition of DCLK1 without

affecting that of normal intestinal organoids. These data suggest that miR-137 regulates the tumorigenic capacity of the colon CSCs through the inhibition of DCLK1 expression, which implies that the miR-137/DCLK1 axis plays an important role in the tumorigenicity of colon CSCs.

As miR-137 has many target genes and off-target effects, we must admit that there is a possibility that the effect of miR-137 on tumorigenicity resulted from the inhibition of another mRNA (9). Actually, some reports have shown that miR-137 has various targets, such as Cdc42, pyruvate kinase isozymes, Formin-like 2, and paxillin, which are related to invasion, the inhibition of the Warburg effect, and metastasis in colorectal cancers, respectively (31–34). In addition, miR-137 is epigenetically regulated through promoter hypermethylation in colorectal cancers and the epigenetic silencing of miR-137 occurs at the early stage of colorectal carcinogenesis (35). Furthermore, it has been shown that DCLK1 has the function to maintain the intestinal regeneration and regulate the Wnt signaling pathway in the DSS-induced colonic mouse model (36). In addition to these reports, our findings provide compelling evidence that miR-137 could be a key regulator of colon CSC properties, and a promising therapeutic factor for colon cancer without affecting the growth of normal colon tissues.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Sakaguchi, S. Hisamori

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Sakaguchi, S. Hisamori, N. Oshima

Writing, review, and/or revision of the manuscript: M. Sakaguchi, S. Hisamori, N. Oshima, F. Sato

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Sakaguchi, S. Hisamori, N. Oshima, Y. Shimono

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