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MicroRNA 133A Regulates Squalene Epoxidase Expression in Colorectal Cancer Cells to Control Cell Proliferation and Cholesterol Production

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Abstract: Background/Objectives: Colorectal cancer (CRC) is one of the most common cancers worldwide, with high incidence and mortality rates. MicroRNAs are endogenous and non-coding RNAs that play a pivotal role in the development and progression of various cancers by targeting specific genes. Previously, we identified *MIR133A* to be significantly decreased in human CRC tissues. This study aims to identify the relationship with *SQLE*, one of the candidate target genes of *MIR133A*, and study their interaction in CRC cells. Methods: Through the luciferase reporter assay, quantitative RT-PCR (qRT-PCR), and Western blot analysis. Results: We identified *SQLE* as a direct target gene of *MIR133A*. Using the MIR133A KI cell lines, which knocked-in *MIR133A1* or *MIR133A2* in CRC cell lines, and CRC cells transfected with *siSQLE*, we found that *MIR133A* regulated the proliferation and migration of CRC cells by modulating SQLE-mediated PIK3CA-AKT1 and CYP24A1 signaling. We also found that cholesterol production was regulated by *MIR133A* in CRC cells. Conclusions: Our results suggest that *MIR133A* is an important therapeutic target for colorectal cancer.

Keywords: colorectal cancer; *MIR133A*; SQLE; cholesterol

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, its frequency increasing in developed countries [1]. Accumulating research has shown that genetic alterations, epigenetic alterations, age, diet, smoking, alcohol, microbiome, and a lack of exercise are associated with CRC [2,3]. Targeted therapies based on the results of many molecular studies on CRC have improved the survival rates, but they are still limited. Recently, microRNA (miRNA, MIR)-based gene therapy has emerged as a novel strategy for cancer treatment, as many miRNA target sites have been found to be localized in cancer-associated genomic regions [4,5]. Therefore, an understanding of miRNAs regulating molecular mechanisms through the post-transcriptional repression of genes in CRC tumorigenesis is needed.

An miRNA is a small, non-coding RNA molecule of about 22 nucleotides that binds to the 3′-untranslated region (UTR) of the target messenger RNA (mRNA) to regulate gene expression at the post-transcription level [6]. mRNA plays an important role in the regulation of various biological processes, such as cell proliferation, migration, apoptosis, angiogenesis, and the development of numerous types of cancer [7,8]. Many miRNAs and their target genes have been discovered, and miRNAs provide new and promising methods for cancer diagnosis, prognosis, and treatment [9,10]. Recent reports have shown that microRNAs are also present in feces, and attempts are being made to correlate CRC with fecal microRNAs [11,12]. miRNA-based treatments are in the early stages of development, and further research is needed to evaluate their efficacy and safety in the clinical environment.

Accumulating studies, including those on microRNA 133A (also known as *MIR133A*, *has-miR-133A*), have demonstrated that miRNA is associated with the development of CRC [13,14]. There are three known miRNA133 genes in the human genome: *MIR133A1*, *MIR133A2*, and *MIR133B*, found on chromosomes 18, 20, and 6, respectively. The mature sequences of *MIR133A1* and *MIR133A2* are the same and differ by one sequence from that of *MIR133B*. *MIR133A* plays an important role in advancing malignant tumors such as CRC, bladder, breast, and prostate cancer [15]. Several studies have shown decreased *MIR133A* expression in non-small-cell lung cancer [16], bladder cancer [17], prostate cancer [18], and lung squamous epithelial cell cancer [19]. However, as *MIR133A* has been reported to increase in breast cancer [20], it is thought to show tissue-specific expression patterns.

Previously, we showed that *MIR133A* was downregulated in CRC tissue [21] and also suggested that *MIR133A* regulates proliferation, migration, and apoptosis by inhibiting SOX9 and CDH3 expression in CRC cells [21,22]. We compared the transcriptome analysis data results of *MIR133A1* knock-in (*MIR133A1* KI) cells and *MIR133A2* KI cells with the candidate target genes of *MIR133A1* and *A2* predicted by bioinformatic tools and identified 29 and 33 candidate target genes of *MIR133A1* and *A2*, respectively [21]. The squalene epoxidase (*SQLE*) gene was one of the candidates for the *MIR133A* target gene.

In this study, we found that *SQLE* is a direct target gene of *MIR133A* and demonstrated the association of *MIR133A* and SQLE in human CRC cells and tissues. We also demonstrated in CRC cells that *MIR133A* suppresses SQLE expression, which regulates cell proliferation and migration as well as cholesterol production.

2. Materials and Methods

2.1. Patient Samples

Tissue samples were obtained from 12 patients with CRC (7 females, 5 males), 4 each from T2, T3, and T4 tumor stages, from the Biobank of Wonkwang University Hospital, which provided tumor tissue and adjacent healthy tissue. Informed consent from the subjects was obtained by the biobank, and this study was approved by the institutional review board (WKIRB-202006-BR-023). The tissues were used to quantify SQLE protein by Western blot. The average age of the patients with CRC was 74.1 years. Further information can be found in Supplementary Table S1.

2.2. Cell Culture

The human normal colon cell line CCD841 CoN and CRC cell lines (HCT116 and SW48) were purchased from the Korea Cell Line Bank (Seoul, Korea) or the American Type Culture Collection (Rockville, MD, USA). SW48 and HCT116 cells were cultured according to the methods of previously published papers [22,23]. CCD841 CoN cells were cultured in EMEM (Hyclon, Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS) at 37 °C in 5% CO_2 in a humidified atmosphere. MIR133A1 KI or MIR133A2 KI cell lines overexpressing MIR133A1 or MIR133A2 were generated in stable Tet-on advanced HCT116 or SW48 cell lines using 500 µg/mL G418 (Takara Bio, Kusatsu, Japan), with a maintenance concentration of 100 µg/mL G418.

2.3. RNA Extraction and Quantitative Real-Time PCR

RNA extraction and qRT-PCR were performed using methods previously reported in [24]. Quantification of the maturation level of MIR133A was performed using the TaqMan miRNA assay. SYBR green dye (Applied Biosystems, MA, USA) was used to assess mRNA expression in qRT-PCR. RNU48 (for TaqMan qRT-PCR) and GAPDH were used as endogenous controls for miRNA and mRNA qRT-PCR, respectively. Each sample was analyzed in triplicate. The primers used in this study are listed in Supplementary Table S2.

2.4. MIR133A Transfection

HCT116 cells, SW48 cells (both 3×10^5 cells/well), and CCD841 CoN cells were plated on 10 cm dishes and cultured as described above. The *MIR133A* mimic (*hsa-miR-133A*, pre-miR miRNA precursor AM17100, product ID: PM12946) and negative control oligonucleotides were commercially synthesized (Ambion, Austin, TX, USA) and used at 50 nmol/mL for transfections. Transfections were performed with lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) or siPORT NeoFX transfectants (Ambion), respectively, according to the manufacturer's recommended methods. *SQLE* small interfering RNA (*siSQLE*) and negative control siRNA transfections were also performed according to the manufacturer's protocol (Ambion). After transfection, the cells were cultured and harvested for 48 or 72 h for mRNA or protein analysis, respectively.

2.5. Plasmid Construct and Luciferase Assay

Luciferase assays were performed according to previously published methods [25]. Wild-type (WT) or mutant (MT) constructs of the *SQLE 3′-UTR* were generated by PCR using the primers listed in Table S1. They were then ligated into the pmirGLO dual-luciferase miRNA-targeting expression vector (Promega, WI, USA). All constructs were verified by DNA sequencing. For this assay, samples of 5×10^4 cells/well were inoculated into 24-well plates and transfected with *SQLE* WT or MT constructs or *MIR133A* or *MIRNC* (negative control) using lipofectamine (Invitrogen) and siPORTTM NeoFXTM transfectants (Ambion). Twenty-four hours later, the bioluminescence of Renilla and fireflies was measured using the Dual-Glo luciferase assay system (Promega). This experiment was repeated three times.

2.6. MTT Cell Viability and Migration Assay

To determine the effect of *MIR133A* on the cell viability of CRC cell lines, HCT116 (8×10^3 cells/well) and SW48 (1×10^4 cells/well) were transfected in 96-well plates with the *MIR133A* mimic (50 nM), siSQLE, or the control and incubated in humidified air containing 5% CO_2 at 37 °C for the specified time. Further steps were performed according to our previous method [23].

2.7. Western Blot Analysis

Western blot analysis was performed according to our previously published methods [23,25]. The primary antibodies used were the following: SQLE (PA5-76523) and CYP24A1 (PA5-21704) from Thermo Fisher Scientific Inc. (Waltham, MA, USA); pAKT1 (#9271), pERK1/3 (#9101), and pGSK3β (#9336) from Cell Signaling Technology; GAPDH (sc-47724) and PIK3CA (sc-293172) from Santa Cruz Biotechnology (Dallas, TX, USA); and CTNNB1 (610153) from BD Biosciences (Franklin Lakes, NJ, USA). Protein quantification was performed using ImageJ software 1.54, and GAPDH was used as a control. All experiments were repeated at least three times.

2.8. Quantification of Total Cholesterol in Cells Overexpressing MIR133A

Cholesterol was extracted from *MIR133A1* KI and *MIR133A2* KI cells and from CCD841 cells transfected with *MIR133A* by methanol and chloroform (vol/vol, 1:2) and was measured using the Cholesterol Quantification Assay kit (Cat#CS0005; Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer′s instructions. Experiments were repeated three times.

2.9. Statistical Analysis

All experiments in this study were performed at least three times using independent data sets. Data are presented as the mean ± standard deviation (SD). All statistical analyses were performed using Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 8 (one-way analysis of variance [ANOVA]). Two-tailed Student's t-tests or Tukey's tests were used to compare multiple data sets. A p value < 0.05 was considered statistically significant.

3. Results

3.1. SQLE Is a Direct Target of MIR133A

The *3′-UTR* of *SQLE* was expected to interact with *MIR133A*. To evaluate the interaction of the *SQLE 3′-UTR* with *MIR133A1*, we constructed the pmirGLO-*SQLE* WT luciferase vectors (Figure 1A). Compared to the negative control, luciferase activities were significantly decreased when the cells were co-transfected with the *MIR133A* mimic (Figure 1B). Additionally, as a control group, mutant-type MT *SQLE 3′-UTR* was constructed by deleting the complementary bases of *MIR133A*. As expected, the inhibition of luciferase activity by *MIR133A* was not detected in the mutant binding site (Figure 1B). We examined the role of *MIR133A* in *SQLE* mRNA and protein expression in HCT116 and SW48 cells. For that, we performed qRT-PCR to measure the expression level of *SQLE* mRNA in *MIR133A* mimic-transfected cells. The *SQLE* mRNA level was significantly decreased compared to the mock normal conditions (Figure 1C, $p < 0.001$). Likewise, cellular SQLE protein was downregulated considerably in *MIR133A*-transfected cells (Figure 1D, $p < 0.05$). Collectively, these results indicate that *SQLE* is a direct target of *MIR133A*.

3.2. SQLE Expression Level in MIR133A Knock-In Cell Lines

We investigated *SQLE* mRNA and protein expression levels in *MIR133A1* KI and *MIR133A2* KI cells. To confirm this, we performed qRT-PCR to measure the expression levels of *SQLE* mRNA in MIR133A1 KI and MIR133A2 KI cells and Western blot to measure protein expression. The results showed that the SQLE mRNA level was significantly reduced in *MIR133A1* KI and *MIR133A2* KI cells compared to the mock normal conditions (Figure 1E). Similarly, SQLE protein expression was significantly downregulated in the *MIR133A1* KI and *MIR133A2* KI cell lines that knocked-in *MIR133A1* and *MIR133A2* into the HCT116 and SW48 cell lines, respectively (Figure 1F).

3.3. MIR133A Regulates SQLE-Mediated PIK3CA-AKT1 Signaling

Based on the above results, we evaluated whether SQLE regulation by *MIR133A* or *siSQLE* affects the PIK3CA-AKT1 signaling pathway in *MIR133A1* KI and *MIR133A2* KI cells. The Western blot results revealed that phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA, also known as PI3K) and AKT serine/threonine kinase 1 (AKT1) were significantly downregulated by *MIR133A* overexpression in HCT116 and SW48 (Figure 2A). We also examined the AKT1 downstream pathways of glycogen synthase kinase 3 beta (GSK3B, also known as GSK-3β) and catenin beta 1 (CTTNB1, also known as β-catenin) in *MIR133A1* KI and *MIR133A2* KI cells. Our Western blot results showed that GSK3B expression was increased but CTTNB1 expression was decreased in both MIR133A KI cell lines (Figure 2A). Similar results were obtained when we knocked-down *SQLE* in both HCT116 and SW48 cell lines via *siSQLE* transfection (Figure 2B).

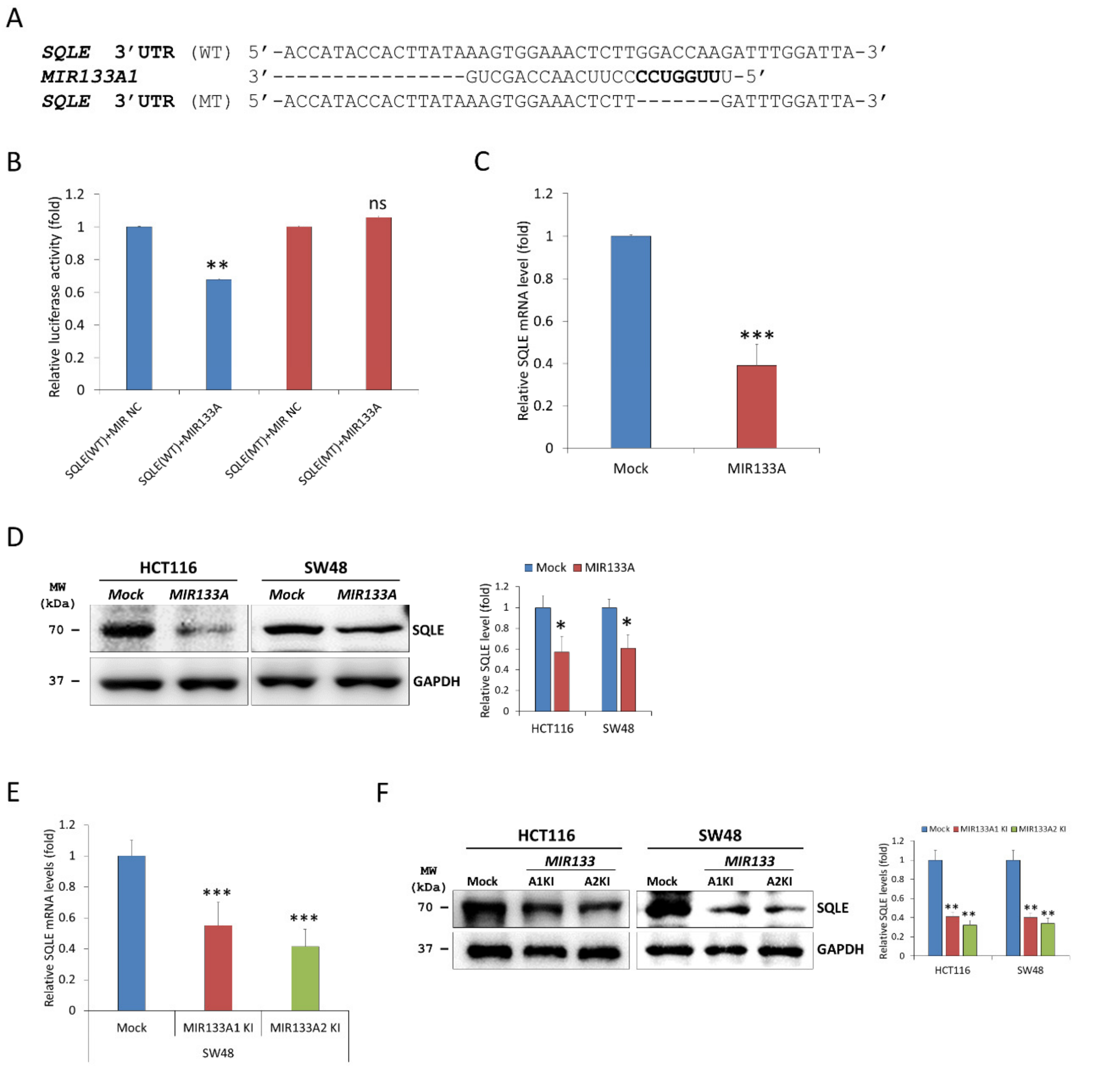

Figure 1. *SQLE* is a direct target of *MIR133A*. (**A**) *ATOX* wild-type (WT) and mutant-type (MT) constructs were cloned into the pmirGLO vector. (**B**) The cloned pmirGLO vector with WT or MT *SQLE* 3′-UTR was transfected into HCT116 cells, and luciferase activity was measured using a dual-luciferase assay. The results are shown as relative firefly luciferase activity normalized to Renilla luciferase activity. (**C**) qRT-PCR analysis of *SQLE* mRNA in HCT116 and SW48 cells after transfection with NC and *MIR133A*. Data are shown as a fold change with respect to the mock. (**D**) Western blot analysis of cellular SQLE protein levels in HCT116 and SW48 cells after transfection with *MIRNC* or *MIR133A*. After 72 h of transfection, the SQLE protein was collected. (**E**) qRT-PCR analysis of *SQLE* mRNA expression in *MIR133A1* and *A2* KI SW48 cells relative to the mock cells. (**F**) Western blot analysis of SQLE in *MIR133A1* and *A2* KI SW48 and HCT116 cells. NC, normal control (mock). Data were obtained from three independent experiments, and differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control (ns = not significant).

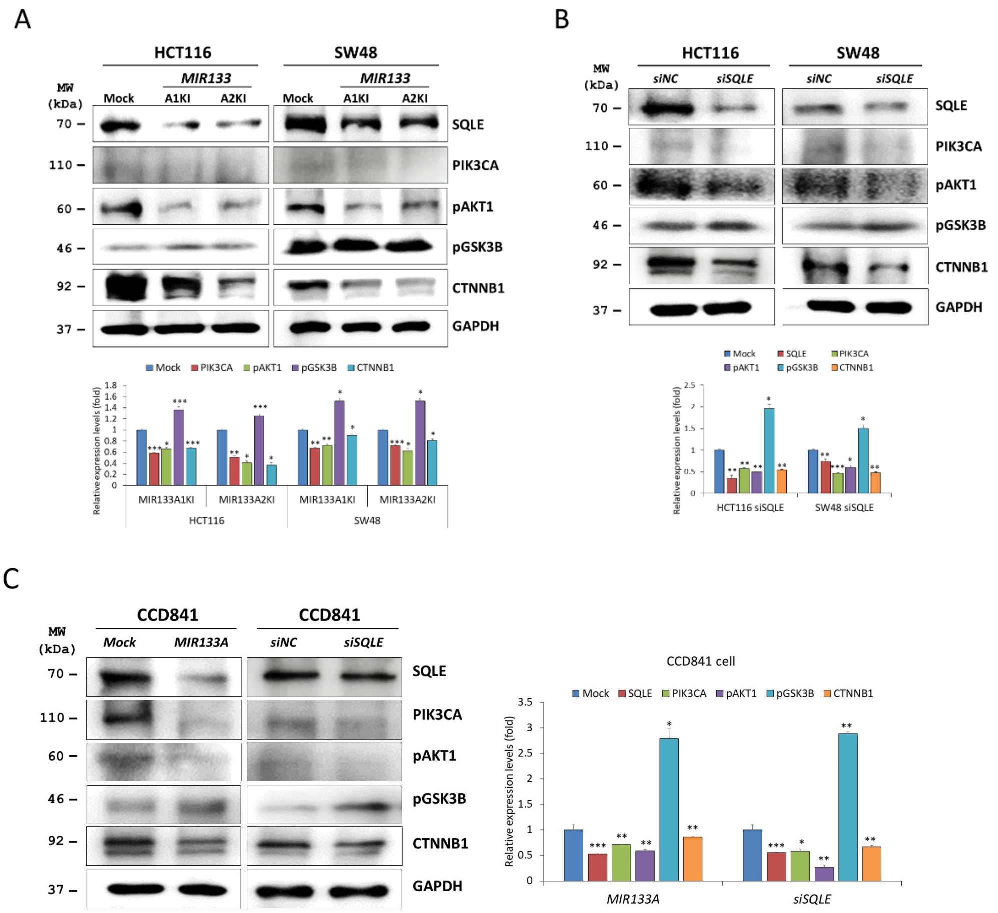

Figure 2. MIR133A regulates SQLE and its downstream PIK3CA pathway molecules. (**A**) Western blot analysis of SQLE and its downstream PIK3CA, pAKT1, pGSK3B, and CTNNB1 in MIR133A1 KI and MIR133A2 KI cells overexpressing MIR133A1 and MIR133A2 in HCT116 and SW48 cells, respectively. (**B**) Western blot analysis of SQLE and its downstream PIK3CA-pAKT1-GSK3B-CTNNB1 in HCT116 and SW48 cells transfected with *siSQLE*. (**C**) SQLE and its downstream PIK3CA-pAKT1-GSK3B-CTNNB1 in CCD841 cells transfected with MIR133A or *siSQLE*. The protein levels of the respective genes were determined by Western blot and densitometry using Image J, where GAPDH was used as a loading control. The representative data from at least three independent experiments are shown. Each bar represents the mean fold alternation above or below the control (±S.D). Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control.

We also investigated whether the *MIR133A*-induced downregulation of SQLE regulated PIK3CA-AKT1 signaling in normal colon cell lines. As shown in Figure 2C, transfection of the CCD841 cell line with *MIR133A* significantly decreased the expression of SQLE and of its downstream signal PIK3CA-AKT1 (Figure 2C). To determine whether PIK3CA-AKT1 signaling was due to the regulation of SQLE by *MIR133A*, we transfected CCD841 cells with *siSQLE* to measure the expression of PIK3CA-AKT3 and obtained the same results as the *MIR133A* transfection experiment (Figure 2C). These results suggest that *MIR133A* regulates the SQLE-mediated PIK3CA-AKT1 signaling pathway in normal colon cells as well as in CRC cells.

3.4. MIR133A Regulates SQLE-Mediated CYP24A1 Signaling in CRC Cells

A recent study reported that SQLE promotes CRC cell proliferation through the activation of cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*)-mediated MAPK signaling [26]. This information led us to investigate whether SQLE, the expression of which is downregulated by *MIR133A*, affects CYP24A1-mediated MAPK signaling. Our results revealed that CYP24A1 and its downstream molecule, mitogen-activated protein kinase 1 (*MAPK1*, also known as *ERK*), were significantly downregulated by *MIR133A* overexpression in both *MIR133A1* KI and *MIR133A2* KI cells (Figure 3A). To determine whether the regulation of CYP24A1-MAPK1 expression by *MIR133A* is SQLE-mediated, we transfected the HCT116 and SW48 cell lines with *siSQLE* to examine the expression of CYP24A1 and MAPK1. As shown in Figure 3B, we obtained similar results when we suppressed the expression of *SQLE* in both HCT116 and SW48 cells by introducing *siSQLE* (Figure 3B).

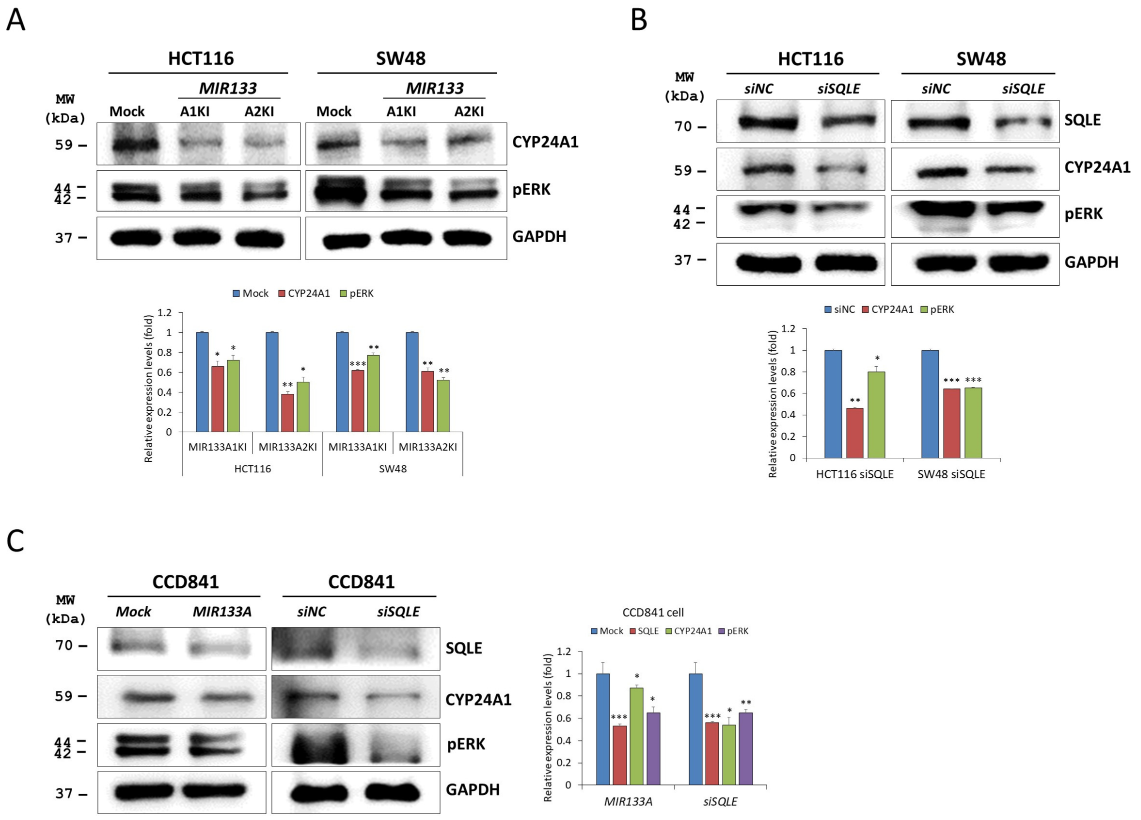

Figure 3. *MIR133A* regulates SQLE-mediated downstream CYP24A1 pathways. (**A**) Western blot analysis of SQLE and downstream CYP24A1 and pERK in *MIR133A1* KI and *MIR133A2* KI cells overexpressing *MIR133A1* and *MIR133A2* in HCT116 and SW48 cells, respectively. (**B**) Western blot analysis of SQLE and its downstream CYP24A1 and pERK in HCT116 and SW48 cells transfected with *siSQLE*. (**C**) SQLE and its downstream CYP24A1 and pERK in CCD841 cells transfected with *MIR133A* or *siSQLE*. The protein level of each gene was determined by Western blot and densitometry using Image J, where GAPDH was used as a loading control. Representative data from at least three independent experiments are shown. Each bar represents the mean fold change above or below the control (±SD). Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the mock control.

We also investigated whether the downregulation of SQLE by *MIR133A* regulates CYP24A1-MAPK1 signaling in CCD841 cells. We found that transfection of the CCD841 cell line with *MIR133A* significantly reduced the expression of SQLE and decreased the expression of its downstream signal, CYP24A1-MAPK1 (Figure 3C). To confirm that CYP24A1-MAPK1 signaling was due to SQLE regulation, we transfected CCD841 cells with *siSQLE* to measure the expression of CYP24A1-MAPK1 and obtained similar results (Figure 3C). These results suggest that *MIR133A* regulates the SQLE-mediated CYP24A1-MAPK1 signaling pathway in CRC cells.

3.5. MIR133A Inhibits SQLE-Mediated Cell Proliferation in CRC Cells

The above results imply that *MIR133A* affects cell proliferation in CRC cells. This led us to perform the MTT assay to investigate whether *MIR133A* affects cell proliferation by regulating the expression of SQLE. The results showed that HCT116 and SW48 cell lines overexpressing *MIR133A* had significantly inhibited cell proliferation compared to the control, and the same results were obtained when these cell lines were transfected with *siSQLE* (Figure 4A). These results suggest that *MIR133A* inhibits cell proliferation in CRC cell lines by targeting *SQLE*.

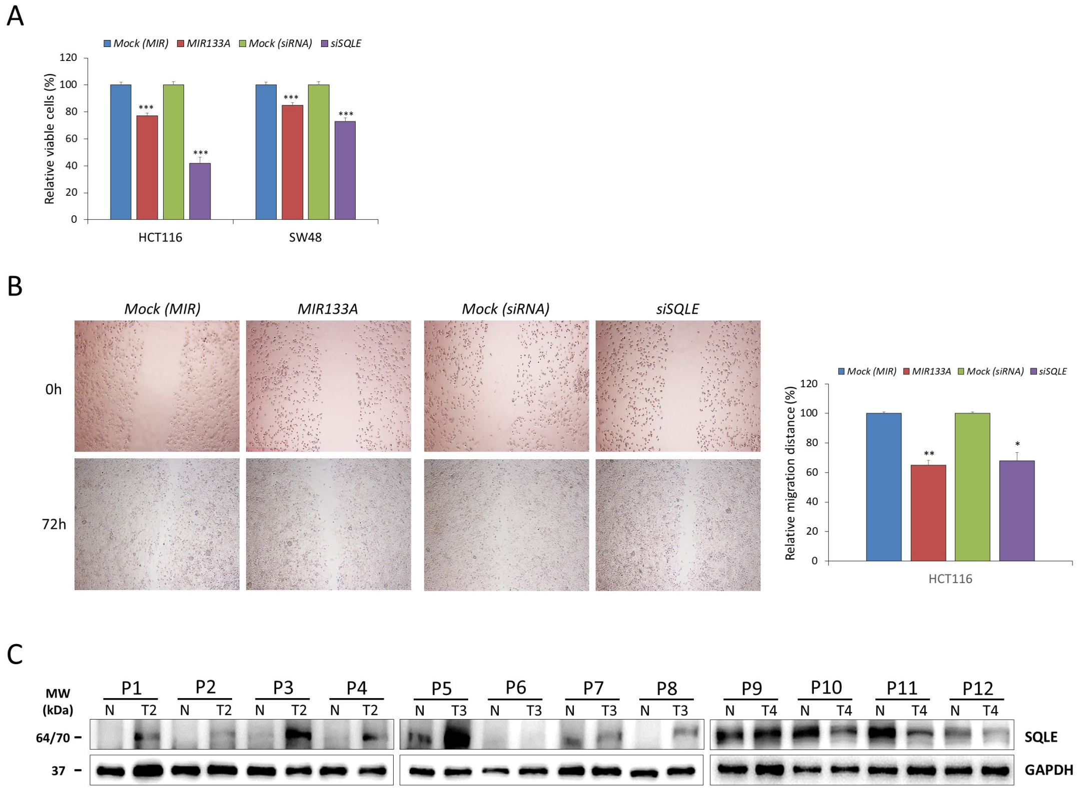

Figure 4. *MIR133A* inhibits cell proliferation and cell migration by targeting SQLE in CRC cell lines. (**A**) MTT assays of *MIR133A* mimic- or *siSQLE*-transfected HCT116 and SW48 cell lines. (**B**) The scratch wound assay was conducted in *MIR133A* mimic-transfected or *siSQLE*- transfected HCT116 cells. The migration distance was measured at 0 and 72 h after the cells were scratched. Representative data from at least three independent experiments are shown. Each bar represents the mean fold change above or below the control (±SD). Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the mock control. (**C**) SQLE expression in human CRC tissues. SQLE protein expression in 12 CRC tissue samples and adjacent normal tissues (4 samples each at T2, T3, and T4 stages). All tissues were from patients with CRC. The Western blotting results were desensitized using Image J, and GAPDH was used as a loading control.

3.6. MIR133A Inhibits SQLE-Mediated Cell Migration

To investigate whether *MIR133A* affects cell migration by regulating the expression of SQLE, we performed a scratch wound assay. The migration ability of *MIR133A*-overexpressing HCT116 cells was significantly inhibited 72 h after a wound scratch ($p < 0.01$, Figure 4B), and the HCT116 cells transfected with *siSQLE* showed similar results, with their migration ability significantly inhibited 72 h after transfection ($p < 0.05$, Figure 4B). These data suggest that *MIR133A* inhibits CRC cell migration by regulating SQLE.

3.7. SQLE Expression in Human CRC Tissues

Our previous report showed that *MIR133A* is downregulated in human CRC tissues [18], so we expected that a decrease in *MIR133A* in human CRC tissue would result in an increase in the product of its target gene, SQLE. To confirm this, we evaluated SQLE expression in 12 human CRC tissues (4 each at CRC stages T2, T3, and T4) and adjacent normal tissues by Western blot analysis. We found that the expression of the SQLE protein was upregulated in tumor tissues at stage T2 compared to adjacent normal tissues and gradually decreased as they progressed to stage T4 (Figure 4C).

3.8. MIR133A Regulates SQLE-Mediated Cholesterol Production

SQLE is one of the rate-limiting enzymes involved in cholesterol synthesis and is well known for catalyzing the first oxygenation step in the conversion of squalene to 2,3(S)-monooxy dosqualene (MOS). To determine whether cholesterol synthesis in CRC cells is regulated by *MIR133A*, we examined cholesterol expression in the *MIR133A1* KI and *MIR133A2* KI cell lines, in which either *MIR133A1* or *MIR133A2* was overexpressed in the HCT116 and SW48 cell lines, respectively. As shown in Figure 5A, the expression of cholesterol was significantly reduced in the *MIR133A* KI cell lines compared to the control (Figure 5A). This reduction in cholesterol was also observed in the CCD841 cell lines transfected with *MIR133A1* (Figure 5B). These results suggest that *MIR133A* may play an important role in cholesterol synthesis in the normal colon and CRC.

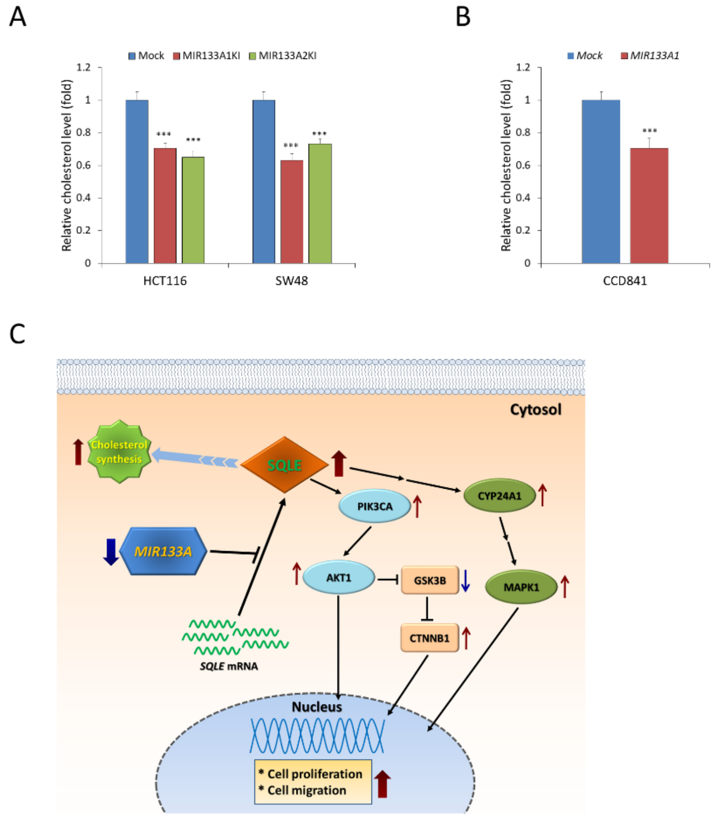

Figure 5. Cholesterol content analysis and simple putative mechanism of *MIR133A*. (**A**) Cholesterol content analysis in *MIR133A1* KI and *MIR133A2* KI cells overexpressing *MIR133A1* and *MIR133A2* in HCT116 and SW48 cells, respectively. (**B**) Cholesterol levels in CCD841 cells transfected with *MIR133A*. Each bar represents the mean fold change above or below the control (±SD). Differences were considered statistically significant at *** $p < 0.001$ compared to the mock control. (**C**) Simple putative mechanism of *MIR133A* regulation of SQLE-mediated cell proliferation, colony formation, and cholesterol synthesis in human CRC.

4. Discussion

CRC is the third most common cancer in the world and has high morbidity and mortality. CRC treatment has developed significantly, but it is still limited, and the prognosis of CRC is poor due to recurrence and metastasis. Therefore, it is important to identify the molecular mechanisms and novel genes involved in the development of CRC to create more effective therapies. One effective therapeutic approach is the use of microRNAs. Endogenous miRNAs in the human genome are highly stable and precisely regulate the expression of a variety of genes. In addition, an abnormal lipid metabolism is increasingly considered a hallmark of many cancers, so it is important to identify microRNAs that target genes involved in lipid metabolism in CRC to understand their function. Previously, we showed that *MIR133A* is downregulated in CRC tissues and suggested that the *SQLE* gene is one of the candidate target genes of *MIR133A* [21].

SQLE is a pivotal mammalian cholesterol synthesis enzyme that converts squalene to 2–3 oxidosqualene, ultimately leading to the synthesis of sterol and cholesterol [27]. An imbalance in cholesterol biosynthesis is linked to carcinogenesis [28,29]. SQLE is expressed abnormally in melanoma, CRC, glioma, breast cancer, renal cancer, and ovarian cancers [27,30]. SQLE plays a critical role in promoting tumor development and migration in squamous cell carcinoma [27,31], CRC [30], and breast cancer [32,33]. Accumulating research has shown that SQLE plays an important role in the initiation and progression of cancer and is a promising target for cancer treatment [34]. In this study, we showed that *SQLE* is a direct target gene of *MIR133A*, which regulated SQLE mRNA and protein levels in CRC cells (Figure 1A–D).

The reprogramming of cholesterol metabolism is a hallmark of many cancer types and has been identified to promote tumor initiation and progression [35]. It has been reported that SQLE can promote tumor growth through cholesterol/cholesteryl ester accumulation and the subsequent activation of several oncogenic pathways, such as PI3K/AKT signaling [36]. Therefore, we investigated whether PIK3CA-AKT1 expression is regulated by SQLE, a direct target of *MIR133A* in CRC, using the *MIR133A* KI cell line and *siSQLE* transfection. Our results showed that *MIR133A* modulated the expression of PIK3CA-AKT1 signaling by changing the expression of SQLE (Figure 2). We also examined the expression of the AKT1 downstream pathway, GSK3B, and CTTNB1, using the *MIR133A* KI cell line and CRC cells transfected with *siSQLE*. The results showed that the overexpression of *MIR133A* increased GSK3B expression but decreased CTTNB1 expression; we obtained similar results when transfecting CRC cells with *siSQLE* (Figure 2A,B). Similar results were obtained when we overexpressed *MIR133A* in CCD841 cells, which are normal colonic epithelial cells (Figure 2C). These results indicate that *MIR133A* regulates PIK3CA-AKT1 signaling by modulating the expression of SQLE in CRC cells and normal intestinal cells.

Extracellular signal-regulated kinase (ERK), an important oncogenic signaling molecule in several cancer types, has been reported to be regulated by SQLE [26,27,37]. In CRC, decreased SQLE was reported to decrease the CYP24A1 level by reducing calcitriol, which inhibits phosphorylation and the activation of ERK [26]. In this study, we found that the expression of CYP24A1 and MAPK1 was reduced in *MIR133A1* KI cell lines overexpressing *MIR133A* and in CRC cell lines transfected with *siSQLE* (Figure 3A,B). We obtained similar results when we overexpressed *MIR133A* by transfecting CCD841 cells, normal colonic epithelial cells, with *MIR133A* (Figure 3C). These results suggest that *MIR133A* regulates the SQLE-mediated CYP24A1-MAPK1 signaling pathway in both normal and CRC cells.

Based on the above results and previous reports [26,38], SQLE promotes cell proliferation in CRC cells. This led us to investigate whether the regulation of SQLE expression by *MIR133A* in CRC cells affects cell proliferation and cell migration. We examined cell growth and migration ability in MIR133A KI cells overexpressing *MIR133A* and in HCT116 and

SW48 cells infected with *siSQLE*. Cell growth was significantly reduced in the MIR133A KI cells and CRC cells transfected with *siSQLE* (Figure 4A). Similarly, cell migration ability was significantly reduced in *MIR133A*-overexpressing HCT116 cells and CRC cells infected with *siSQLE* (Figure 4B). These results demonstrate that *MIR133A* is involved in the growth and migration of CRC cells through the regulation of SQLE expression.

Recent studies have reported that SQLE is overexpressed in CRC [26,30,38,39], and SQLE upregulation is associated with a poor CRC prognosis [26]. However, its potential role in CRC initiation and progression remains controversial. SQLE has been proposed to be an oncogenic factor by promoting cell growth [26], while it has also been argued that a reduction in SQLE can accelerate CRC metastasis [30]. Since our previous report showed that *MIR133A* is downregulated in human CRC tissues [21], we thought that the reduction in MIR133A in human CRC tissues would increase the expression of its target gene, SQLE. To confirm this, we evaluated SQLE expression in 12 human CRC tissues at different CRC stages and adjacent normal tissues and found that the expression of the SQLE protein was upregulated in tumor tissues at stage T2 compared to adjacent normal tissues and gradually decreased with progression to stage T4 (Figure 4C). Despite being based on a small number of samples, these results suggest that SQLE expression is regulated by *MIR133A* in colon cancer initiation and progression. We think that investigators could generalize the findings of SQLE upregulation by *MIR133A* in CRC development and progression through further studies.

Since SQLE is a rate-limiting enzyme essential for cholesterol production [40,41], we determined whether cholesterol synthesis is indeed regulated by *MIR133A* in CRC cells. We examined the expression of cholesterol in the *MIR133A* KI cell line and found that it was significantly reduced in MIR133A KI cells overexpressing *MIR133A* (Figure 5A) and in CRC cells transfected with *siSQLE* compared to the control (Figure 5B). These results suggest that *MIR133A* plays an important role in the regulation of cholesterol synthesis in CRC. In this study, we described a limited molecular regulatory mechanism by which SQLE, whose expression is increased by decreased *MIR133A* in CRC tissues, affects cell proliferation and cholesterol synthesis; however, future studies should investigate other detailed molecular mechanisms in these regulatory processes by SQLE.

5. Conclusions

In this study, we found that SQLE is a direct target gene of *MIR133A* and that, in human CRC cells, *MIR133A1* and *MIR133A2* regulate the expression of SQLE, which is involved in cell proliferation and cell migration by controlling PIK3CA-AKT1 and CYP24A1 signaling. In conclusion, the modulation of PIK3CA-AKT1 and CYP24A1 signaling by SQLE, the expression of which is increased with decreased *MIR133A* in CRC tissues, is likely to regulate cell proliferation and cell migration as well as promote cholesterol production (Figure 5C).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/gastroent16010005/s1, Table S1. Clinical characteristics of CRC patients used in this study; Table S2. Primer sequences used for qRT-PCR and luciferase assay in this study.

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Data Availability Statement: All data generated or analyzed during this study are included in this published article.

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