

The MicroRNA-551a/MEF2C Axis Regulates the Survival and Sphere Formation of Cancer Cells in Response to 5-Fluorouracil

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microRNAs regulate a diverse spectrum of cancer biology, including tumorigenesis, metastasis, stemness, and drug resistance. To investigate miRNA-mediated regulation of drug resistance, we characterized the resistant cell lines to 5-fluorouracil by inducing stable expression of miRNAs using lenti-miRNA library. Here, we demonstrate miR-551a as a novel factor regulating cell survival after 5-FU treatment. miR-551a-expressing cells (Hep3B-lenti-miR-551a) were resistant to 5-FU-induced cell death, and after 5-FU treatment, and showed significant increases in cell viability, cell survival, and sphere formation. It was further shown that myocyte-specific factor 2C is the direct target of miR-551a. Our results suggest that miR-551a plays a novel function in regulating 5-FU-induced cell death, and targeting miR-551a might be helpful to sensitize cells to anti-cancer drugs.

Keywords: anti-cancer drug resistance, cell survival, hepatocellular carcinoma, microRNA, sphere formation

INTRODUCTION

Acquisition of resistance to anti-cancer drugs is one of the major obstacles leading to the failure of cancer treatment (Gottesman, 2002; Hasan et al., 2018). Cancer recurrence

and the growth of resistant tumors reduce the survival rate of cancer patients; therefore, it is important to understand the underlying molecular mechanisms of drug resistance. It has been known that resistance to anti-cancer drugs can be acquired by several mechanisms, such as enhanced DNA damage repair, cell death inhibition, drug inactivation, altered cell proliferation, and activation of cancer stem cells (CSCs) (Cree and Charlton, 2017; Housman et al., 2014; Prieto-Vila et al., 2017). Although several efforts have made to overcome the multi-drug resistance of cancer cells, how drug resistance arises and how cancer cells acquire drug resistance have yet been fully elucidated.

microRNAs (miRNAs) are small non-coding RNAs and function as a pivotal regulator in gene expression by inhibiting translation or facilitating the degradation of target mRNAs (Ambros, 2004; Bartel, 2004). miRNAs have been known to be involved in diverse regulation of cellular processes, such as cell growth, death, differentiation, and self-renewal; therefore, differential expression of certain miRNAs is linked to the pathogenesis of diseases (He and Hannon, 2004; McDermott et al., 2011). Accumulating evidence demonstrates that miRNAs execute active roles in regulating the drug resistance of cancer cells by affecting stress response, cell survival, autophagy, and the absorption or digestion of anti-cancer drugs (An et al., 2017; Fojo, 2007; Wang

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et al., 2015). In previous studies, we have established miRNA-overexpressing cell lines resistant to 5-fluorouracil (5-FU), a widely used medication to treat a range of cancers, to find miRNAs regulating the acquisition of drug resistance, and demonstrated the role of miR-200a-3p and miR-195 in 5-FU-induced cell death (Kim et al., 2018; Lee et al., 2017).

In this study, we investigate the mechanism of resistance to 5-FU-induced cell death using one of the resistant clones established in our previous study (Lee et al., 2017). We identified miR-551a as a novel factor regulating cell survival after 5-FU treatment. miR-551a-expressing cells were resistant to 5-FU-induced cell death and inhibition of miR-551a sensitized cell to 5-FU. We showed that miR-551a increased cell survival after 5-FU treatment, by enhancing myocyte-specific factor 2C (MEF2C)-mediated sphere formation. Our results suggest that miR-551a plays a novel function in regulating 5-FU-induced cell death, and that targeting miR-551a might be helpful to sensitizing cells to anti-cancer drugs.

MATERIALS AND METHODS

Cell culture, transfection of plasmids and small interfering RNAs

Stable cell lines expressing miRNAs were established using a Lenti-miR™ Library as described in our previous studies (Kim et al., 2018; Lee et al., 2017). In brief, cells were transduced with Lenti-miR™ Library (Cat. No. PMIRHPLVAHT-1, System Biosciences, USA) (the multiplicity of infection = 10) and incubated with 10 μ M of 5-FU for two weeks to establish the resistant clones. Hep3B cells were maintained with Dulbecco's modified Eagle's medium (Invitrogen, USA) containing 10% fetal bovine serum and 1% antibiotics. All transient transfections including miR-551a precursor, inhibitor (anti-miR-551a) (Bioneer, Korea), the plasmid encoding GFP-tagged MEF2C (pMEF2C) (Sino-Biological, China) and enhanced green fluorescent protein (EGFP) reporter plasmids were performed using Lipofectamine™ 2000 (Invitrogen, USA). EGFP reporters were constructed by inserting 3'UTR of *MEF2C* mRNA into pEGFP-C1 vector (BD Bioscience, Germany) or site-directed mutagenesis.

Identification of miR-551a in the stable clone

Genomic DNA (gDNA) was isolated from the stable clone using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions. The miRNA sequences integrated into genomic DNA were amplified by polymerase chain reaction (PCR) with specific primers and the nucleotide sequence was determined by sequencing as described in Ref. (Kim et al., 2018; Lee et al., 2017).

Cell viability assay

A colorimetric assay using the tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was used to assess cell viability. Cells (1×10^4 cells) were plated on each well of a 96-well plate and treated with 5-FU, oxaliplatin (OXP), cisplatin (CDDP), or doxorubicin (DOX) for 72 h and then further incubated with 0.5 mg/ml of MTT for 3 h. Formazan crystals were solubilized with isopropanol and the absorbance was measured using a Victor 3 microplate

reader (Perkin Elmer, Finland).

RNA analysis

Total RNAs were isolated from cell lines or sphere using TRIzol® Reagent (Life Technologies, USA). For the analysis of mRNA, complementary DNA (cDNA) was synthesized by reverse transcription using a ReverTra Ace® RT Kit (Toyobo, Japan). For miRNA analysis, cDNA was prepared using the MiR-X™ miRNA First-Strand cDNA synthesis kit (Clontech, USA) according to the manufacturer's instructions. The relative abundance of each transcript was assessed by real-time quantitative polymerase chain reaction (RT-qPCR) using the Biorad SYBR Fast qPCR kit (Biorad, UK) and specific primer sets on the StepOne Plus™ system (Applied Biosystems, USA).

Western blotting analysis

Whole cell lysates were prepared using RIPA buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA and 0.1% sodium dodecyl sulfate separated by electrophoresis in SDS-containing polyacrylamide gels (SDS-PAGE), and transferred onto poly(vinylidene difluoride) (PVDF) membranes (Millipore, USA). The blots were incubated with the following antibodies against GFP, MEF2C (Santa Cruz Biotechnology, USA), and β -actin (Abcam, USA), then sequentially incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology, USA). Chemo-luminescent signals were visualized using NEW Clarity™ ECL substrate (Bio-Rad, USA).

Sphere-forming assay

Cells (1×10^3 cells) were seeded in low attachment 96-well plate, and cultured in serum-free medium. After one week, spheres were observed and manually counted. The number of spheres was analyzed in triplicate for each cell type, and at least three independent experiments were carried out.

RESULTS

Hep3B clone expressing miR-551a is resistant to 5-fluorouracil-induced cell death

To identify miRNAs involved in the acquisition of anti-cancer drug resistance to 5-FU, we established stable cell lines expressing certain miRNAs using lenti-miR library with sequential exposure to 5-FU as shown in Fig. 1A (Lee et al., 2017). The specific miRNA expressed in the GFP-positive survival clone was identified as miR-551a by genomic DNA PCR and sequencing analysis of PCR amplicon (Fig. 1B). To analyze the relative expression of miR-551a between miR-551a-expressing clone (Hep3B-lenti-miR-551a) and control cell (Hep3B-lenti-miR-Ctrl), miR-551a level was determined by miRNA RT-qPCR and the result showed higher expression of miR-551a in Hep3B-lenti-miR-551a cell (Fig. 1C). Because Hep3B-lenti-miR-551a clone survived sequential exposure to 5-FU, the relative response of Hep3B-lenti-miR-551a and Hep3B-lenti-miR-Ctrl cells to 5-FU was assessed by MTT assay. Figure 1D shows that the cell viability of Hep3B-lenti-miR-551a cell was higher than that of Hep3B-lenti-miR-Ctrl.

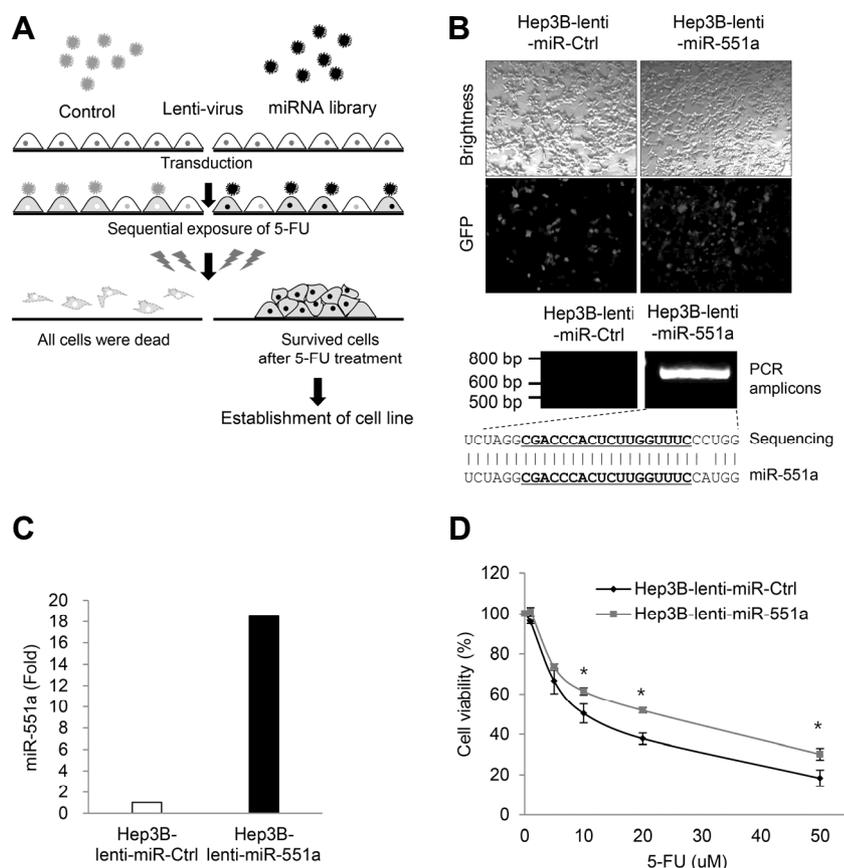


Fig. 1. Hep3B cells stably expressing miR-551a are resistant to 5-FU-induced cell death.

(A) After infection with a lentiviral miRNA library, Hep3B cells were exposed to 10 μM of 5-FU until all control cells (Hep3B-lenti-miR-Ctrl) were dead. Surviving clones were isolated and established as 5-FU-resistant Hep3B clones. (B) GFP expression of Hep3B-lenti-clones was observed using fluorescence microscopy. The insertion of miRNA gene integrated from lentivirus was analyzed by sequencing of PCR products amplified from genomic DNA (gDNA) using specific primers, and identified as miR-551a in Hep3B-lenti-miR-551a clone. (C) Relative levels of miR-551a between Hep3B-lenti-miR-Ctrl and Hep3B-lenti-miR-551a were quantified by miRNA RT-qPCR. U6 RNA was used for normalization. (D) Hep3B-lenti-miR-Ctrl and Hep3B-lenti-miR-551a cells were exposed to 5-FU (1, 5, 10, 20, and 50 μM) for 72 h, and cell viability was determined by MTT assay. Data represent mean \pm SEM from three independent experiments. * $P < 0.05$

These results indicate that Hep3B-lenti-miR-551a cell was resistant after 5-FU treatment, and that miR-551a has a role in the regulation of 5-FU-induced cell death.

miR-551a increases cell viability in response to anti-cancer drugs

To investigate whether miR-551a is an essential factor in regulating cell survival after anti-cancer drug treatment, cell viability between Hep3B-lenti-miR-551a and Hep3B-lenti-miR-Ctrl cells was assessed by MTT assay. No significant change of cell viability of these cell lines at basal level was observed; however, Hep3B-lenti-miR-551a cells had higher viability in response to multiple anti-cancer drugs including 5-FU, oxaliplatin (OXP), cisplatin (CDDP), and doxorubicin (DOX) compared to Hep3B-lenti-Ctrl cells (Figs. 2A and 2B). To determine whether a high level of miR-551a is responsible for an increase of cell survival in Hep3B-lenti-miR-551a cell, we investigated the effect of miR-551a inhibition using anti-miR-551a. Ectopic expression of anti-miR-551a resulted in a significant reduction of cell viability after treatment of 5-FU, CDDP, and DOX (Fig. 2C). These results indicate that miR-551a has a role in promoting cell survival in response to anti-cancer drugs.

miR-551a promotes the sphere formation of Hep3B cells

We observed that Hep3B-lenti-miR-551a cells are resistant to 5-FU-induced cell death, thereby increasing cell survival after

5-FU treatment. Several studies have shown that sphere formation is one of the essential characteristics affecting the drug resistance of cancer cells (Cao et al., 2011; Chen et al., 2012; Ciocce et al., 2010; Fujii et al., 2009). Therefore, we investigated the sphere-forming ability of Hep3B-lenti-miR-551a cells. Figure 3A shows that Hep3B-lenti-miR-551a cells displayed enhanced sphere-forming ability, compared to Hep3B-lenti-miR-Ctrl cells. In addition, the relative expression of common stemness regulators, including *OCT4*, *NANOG*, *KLF4*, and *SOX2*, was slightly upregulated in Hep3B-lenti-miR-551a cells. To further investigate whether miR-551a has the potential to regulate sphere-forming ability, the number of spheres was analyzed after ectopic expression of miR-551a in Hep3B cells. Ectopic expression of miR-551a increased the number of spheres and the levels of common stemness regulators in Hep3B cells (Fig. 3B). In addition, sphere-forming ability between Hep3B-lenti-miR-551a cells and Hep3B-lenti-miR-Ctrl cells after 5-FU treatment was further analyzed. While Hep3B-lenti-miR-Ctrl cells failed to form spheres after 5-FU treatment, Hep3B-lenti-miR-551a cells survived, and generated spheres, even in the presence of 5-FU (Fig. 3C). Taken together, these results indicate that miR-551a promotes the sphere formation of Hep3B cells, thereby increasing resistance to 5-FU-induced cell death.

miR-551a downregulates MEF2C expression

To determine a potential target of miR-551a, *in silico* analysis

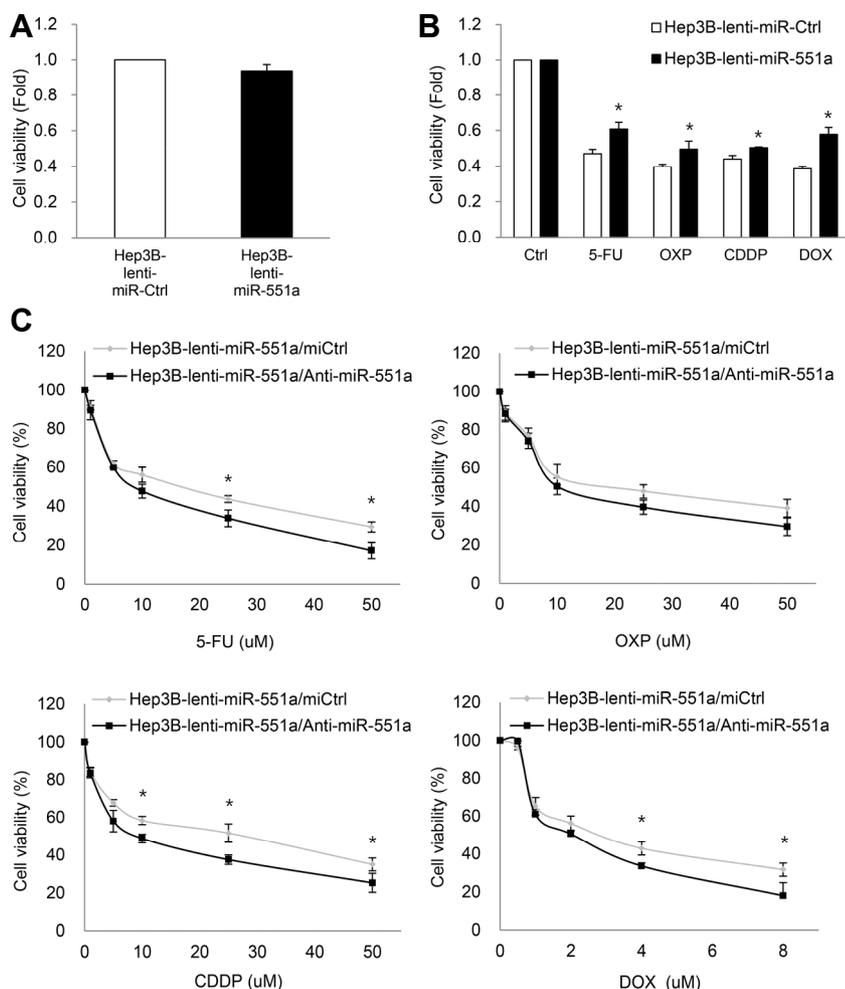


Fig. 2. Inhibition of miR-551a sensitizes Hep3B-lenti-miR-551a cells to several anti-cancer agents. (A and B) Cell viability was determined by MTT assay at the basal level (A), or after incubation with 5-FU, oxaliplatin (OXP), cisplatin (CDDP), and doxorubicin (DOX) for 72 h (B). (C) After transfection of anti-miR-551a or control miRNA, Hep3B-lenti-miR-551a cells were incubated with 5-FU, OXP, CDDP, and DOX for 72 h and cell viability was determined by MTT assay. Data represent mean \pm SEM from three independent experiments. * $P < 0.05$

using Targetscan and miRDB was done and identified *MEF2C* mRNA. Figure 4A shows that *MEF2C* mRNA has the binding site for miR-551a at its 3'UTR (2145-2151 nt). Ectopic expression of miR-551a decreased *MEF2C* expression at both mRNA and protein level in Hep3B cells (Fig. 4B). In addition, a lower expression of *MEF2C* was observed in Hep3B-lenti-miR-551a compared to Hep3B-lenti-miR-Ctrl cell (Fig. 4C). These results indicate that *MEF2C* expression is regulated by miR-551a. To further determine whether *MEF2C* is the direct target of miR-551a, we generated EGFP reporter constructs containing 3'UTR of *MEF2C* mRNA (2015-2246 nt)(pEGFP-*MEF2C* 3U) or mutant 3'UTR missing the seed region binding site for miR-551a (pEGFP-*MEF2C* 3UM) and relative EGFP expression was assessed by Western blotting (Fig. 4D). Overexpression of miR-551a reduced EGFP-*MEF2C* 3U reporter but did not change the level of EGFP control or EGFP-*MEF2C* 3UM (Fig. 4D). These results suggest that *MEF2C* is the direct target of miR-551a.

Knockdown of *MEF2C* enhances the sphere formation of Hep3B cells

We showed that miR-551a-expressing cells increased the number of spheres, and that *MEF2C* is the direct target of

miR-551a (Fig. 3 and Fig. 4). To investigate whether sphere forming ability is affected by *MEF2C*, Hep3B cells were transfected with *MEF2C* siRNA or p*MEF2C* plasmid (Fig. 5A), then cell viability and sphere forming ability were assessed as described previously. Knockdown of *MEF2C* increased, while *MEF2C* overexpression decreased both cell viability and the number of spheres after 5-FU treatment (Figs. 5B and 5C). To further elucidate whether the miR-551a-mediated *MEF2C* downregulation is responsible for the resistant phenotypes in response to 5-FU, *MEF2C* was ectopically expressed in Hep3B-lenti-miR-551a cells and cells were further incubated with 5-FU. As shown in Figs. 5D and 5E, *MEF2C* expression reduced cell viability and the number of spheres in Hep3B-lenti-miR-551a cells after 5-FU treatment. These results indicate that *MEF2C* overexpression sensitized Hep3B-lenti-miR-551a cells in response to 5-FU and the miR-551a-*MEF2C* axis has a role in 5-FU-induced cell death by regulating sphere formation.

DISCUSSION

Although numerous efforts have made to understand how cancer cells acquire anti-cancer drug resistance, it still remains

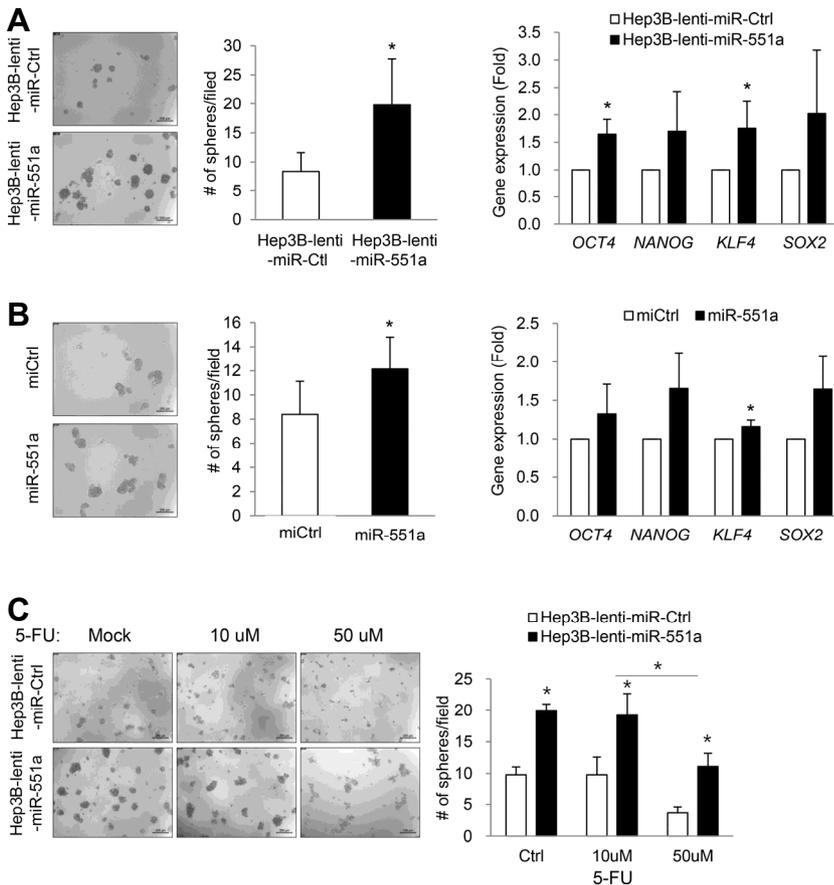


Fig. 3. miR-551a increases the sphere formation of Hep3B cells. (A and B) The number of spheres and relative gene expressions were analyzed in both Hep3B-lenti-miR clones (A) or miR-transfected cells (B). Cells were seeded in a low attachment 96-well plate and cultured in serum-free medium. One week later, the number of spheres was counted from three different areas. Relative levels of *OCT4*, *NANOG*, *KLF4*, and *SOX2* mRNA were analyzed by RT-qPCR. (C) Seventy-two h after 5-FU treatment, the number of spheres and relative gene expression were analyzed between Hep3B-lenti-miR-Ctrl and Hep3B-lenti-miR-551a cells. Images are representative from three independent experiments. Data represent mean \pm SEM from three independent experiments. * $P < 0.05$

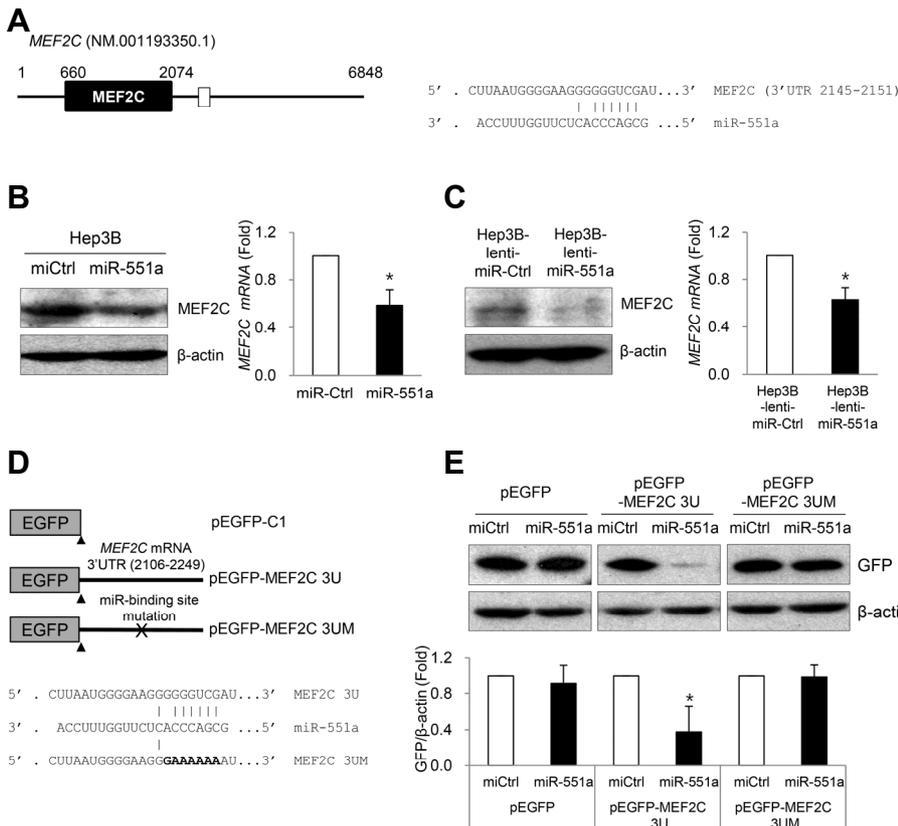


Fig. 4. miR-551a downregulates MEF2C expression. (A) Schematic of *MEF2C* mRNA. miR-551a binding site in *MEF2C* mRNA 3'UTR (2145-2151bp) is shown as a white box. (B and C) Relative expression of MEF2C proteins and mRNA was determined by Western blotting and RT-qPCR in both Hep3B-lenti-miR clones (B) or miR-transfected cells (C). (D) EGFP reporter (pEGFP-MEF2C 3U) containing *MEF2C* mRNA 3'UTR (2106-2249bp) and mutant reporter lacking miRNA binding site were constructed. (E) Hep3B cells were sequentially transfected with miRNAs and EGFP reporters and relative expressions of GFP were determined by Western blotting. β -actin was used as loading control. Images are representative from three independent experiments. Data are mean \pm SEM or representative from three independent experiments. * $P < 0.05$

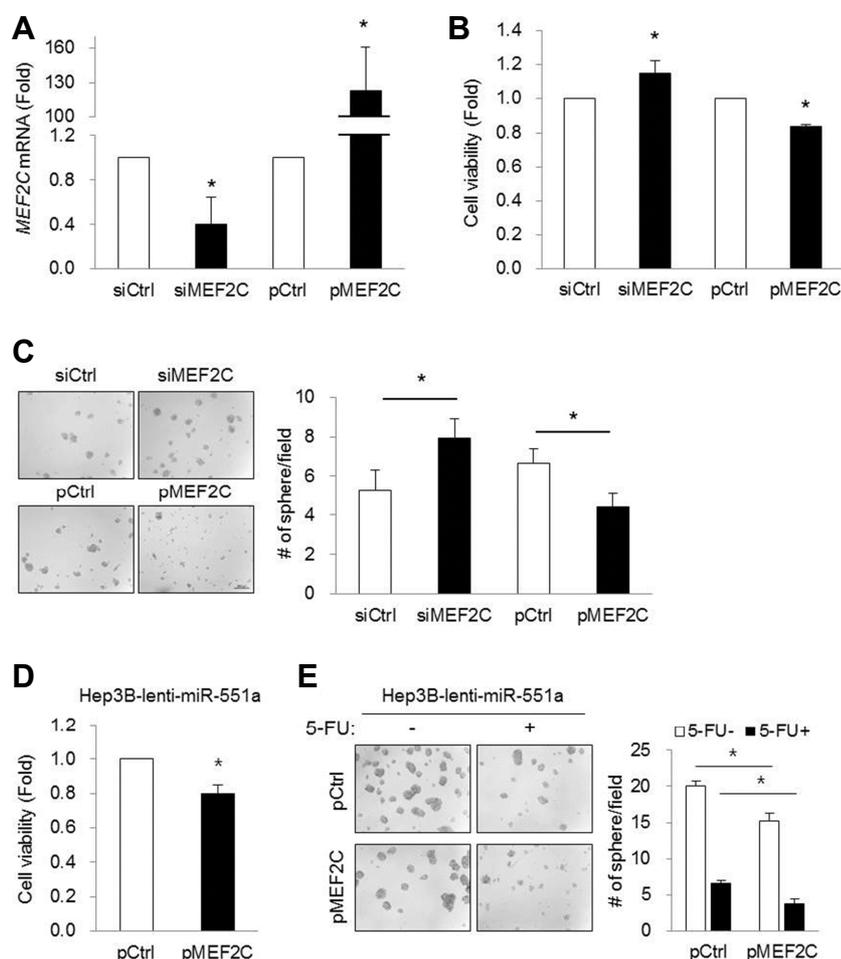


Fig. 5. Knockdown of MEF2C enhances the sphere formation of Hep3B cells. (A-C) Hep3B cells were transfected with MEF2C siRNA or pMEF2C plasmid. Relative levels of *MEF2C* mRNA (A), cell viability (B), and the number of spheres (C) were determined by RT-qPCR, MTT assay, and sphere formation assay after 5-FU treatment. (D and E) After transfection of pMEF2C plasmid, Hep3B-lenti-miR-551a cells were further incubated with 5-FU, then cell viability (D) and the number of spheres (E) were analyzed. Spheres were counted from at least three different fields of each set of experiment. Data are mean \pm SEM from three independent experiments. * $P < 0.05$

a major obstacle to effective cancer therapy. Drug inactivation, drug target alteration, DNA damage response, cell death inhibition, EMT development or an increase of stemness contribute to the anti-cancer drug resistance of cancer cells (Hasan et al., 2018; Housman et al., 2014). In this study, we demonstrate miR-551a as a novel factor regulating cell survival after 5-FU treatment. We identified miR-551a in one of the 5-FU resistant Hep3B clones established using lenti-miR library (Lee et al., 2017). We showed that miR-551a-expressing cells are resistant to 5-FU-induced cell death, and that inhibition of miR-551a sensitized Hep3B cells to 5-FU by regulating MEF2C-mediated sphere formation. Our results suggest that miR-551a plays a novel function in regulating 5-FU-induced cell death, and that targeting miR-551a might decrease the resistance of cancer cells to anti-cancer drugs.

miRNAs execute a major part in several aspects of cancer progression including tumorigenesis, metastasis, differentiation of cancer stem cells, and drug resistance (Caliskan et al., 2017; Deng et al., 2016; Van Roosbroeck and Calin, 2017). Aberrant expressions of certain miRNAs execute tumor suppressive or oncogenic roles, and could be a useful biomarker or drugable target (Hayes et al., 2014; Jansson and Lund, 2012). In this study, we demonstrated the role of miR-551a

as a novel factor regulating anti-cancer drug-induced cell death. miR-551a is known to have a tumor-suppressive function by targeting phosphatase of regenerating liver-3 (PRL-3), insulin receptor substrate 2 (IRS2), and creatin kinase brain-type (CKB) in certain types of cancer, including gastric, colon, and ovarian cancer (Du and Sha, 2017; Li et al., 2012; Loo et al., 2015). Although aberrant expression of miR-551a has been reported so far, little is known about the cellular function and molecular target of miR-551a. We demonstrate that miR-551a promotes cell survival, by increasing the sphere formation of cancer cells after 5-FU treatment (Figs. 2 and 3). In this study, we did not investigate the level of miR-551a in the tissues from the patients who have drug resistance; and further studies are required to provide the clinical significance of miR-551a in the regulation of drug resistance.

MEF2C is a member of the MEF2 gene family and regulates cell differentiation, self renewal, and stemness (Abad et al., 2017; Cho et al., 2011; Ignatius et al., 2017; Li et al., 2008; Rocha et al., 2016). In addition, MEF2C is also known to control cell migration and the proliferation of cancer cells (Wang et al., 2016; Zhang et al., 2018). Therefore, dysregulation of MEF2C expression affects the developmental pro-

cess or cancer progression. Several miRNAs, including miR-21, -27, -135, -155, -204, -214, and -448 have been reported to target *MEF2C* mRNA (Cheng et al., 2018; Chinchilla et al., 2011; Ling et al., 2017; Sahoo et al., 2016; Xu et al., 2015; Yelamanchili et al., 2010; Zhang et al., 2017); and we also showed that miR-551 is one of MEF2C-targeting miRNAs (Fig. 4). We observed that MEF2C directly regulates the sphere-forming ability of Hep3B cells (Fig. 5). The detailed mechanism of MEF2C-mediated sphere formation needs to be further elucidated.

In this study, we characterized Hep3B-lenti-miR-551a cells as a 5-FU resistant clone. The miR-551a-expressing clone survived sequential exposure to 5-FU (Figs. 1 and 2). In Hep3B-lenti-miR-551a cells, sphere-forming ability and the expression of stemness marker genes, which are typical characteristics of cancer-initiating cells (Facompre et al., 2012), were increased even after 5-FU treatment (Fig. 3). These results suggest that miR-551a is the mediator of acquisition of drug resistance, by enhancing the sphere-formation ability of cells. Sphere formation is one of the stem-like properties of cancer-initiating cells, and is known to be related to high levels of self-renewal, tumorigenicity, anti-cancer drug resistance, and invasive phenotype of stem cells (Dieter et al., 2011; Lee et al., 2016). Several studies have demonstrated the essential roles of miRNAs in the regulation of drug resistance, by affecting the sphere-forming ability of cells (Hasegawa et al., 2014; Jeon et al., 2011; Park et al., 2014; Tung et al., 2017; Wang et al., 2017). Therefore, targeting certain miRNAs to reduce the sphere-forming ability of cancer cells could be a useful technique for effective cancer treatment. Our study provides novel insights into the function of miR-551a, which enable to the reduction of anti-cancer drug resistance, thereby increasing the efficiency of chemotherapy.

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