Stony Brook University

miR-128 Inhibits Cell Migration via Downregulation of CEMIP

A Thesis Presented

by

Qian Zhang

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

May 2015

Stony Brook University

The Graduate School

Qian Zhang

We, the thesis committee for the above candidate for the

Master of Science degrees, hereby recommend

acceptace of the thesis

Cao, Jian, M.D., -Thesis Advisor,

Professor, Department of Medicine

Ju, Jingfang Ph.D., -Second Reader, Ph.D

Associate Professor, Department of Pathology

This thesis is accepted by the Graduate School

Charles Taber

Dean of the Graduate School

Abstract of the Thesis

miR-128 Inhibits Cell Migration via Downregulation of CEMIP

by

Qian Zhang

Master of Sciences

in

Biochemistry and Cell Biology

Stony Brook University

2015

Cell Migration Inducing Protein (CEMIP) has been demonstrated to promote cancer progression by enhancing cancer cell migration, hence increasing cancer invasion and metastasis. Upregulated expression of CEMIP in human cancers is associated with poor patient survival rate. Experimental data demonstrated that CEMIP is exclusively detected in human colon cancer cells examined by immunohistochemistry staining. Interestingly, enhanced expression of CEMIP was found in cancer cells located at the invasive front. However, the regulatory mechanism of enhanced CEMIP expression in cancer has not been fully characterized. The aim of this study is to unravel the regulatory mechanism of CEMIP in cancer progression by focusing on post-transcriptional regulation of CEMIP in cancer cells. Employing a bioinformatics tool for identification of potential targeting sites by microRNA (miRNA) within the CEMIP 3'-untranslated region (3'UTR), miRNA -128(1,2) responding elements within the 3'UTR was identified. By surveying human cancer cell lines for CEMIP expression, downregulation of CEMIP was found in aggressive human cancer cell lines and inversely correlated with endogenous CEMIP expression. This observation is in agreement with miR128-1 and CEMIP expression in human colon cancer specimens examined by qPCR. To further determine the correlation of miRNA-128-1 with CMEIP expression, a miR128 inhibitor, called sponge, was generated. When the miR128 sponge was expressed in less aggressive cancer cell lines, CEMIP expression was rescued suggesting specific role of miR128-1 in CEMIP expression. miR128-1 no longer affects CEMIP gene expression once the miR128 binding site within the 3' UTR of CEMIP was mutated. Functionally, overexpression of miR-128-1 in the aggressive cancer cells reduces cell migration. These findings suggest a novel regulatory pathway in invasive cancer cells that overexpression of miR128-1 lead to stabilizing CEMIP mRNA, so expressed CEMIP then induce cancer cell migration.

Acknowledgments

I would like to thank my thesis advisor, Dr. Cao for his generous helping and teaching, and support throughout my thesis work. His guidance and advice have become the critical elements to my success in research and understanding of the project.

I would like to thank Yiyi Li, Jillian Cathcart, Anna Banach, Ashleigh Pulkoski-Gross and Vincent Michael Alford for their assistance in helping me for my thesis project

I would like to thank Prof. Jingfang Ju for reviewing my thesis report, and for his critical comments in helping me to understand and improve my research project.

I would like to thank the department of Biochemistry and Cell Biology for providing me the opportunity to pursue my master degree.

Thank you all!

TABLE OF CONTENTS

List of Figures

Abbreviation

CEMIP= Cell Migration Inducing Protein

miR-128=micro RNA-128

conA= concanavalin A

EMT= Epithelial-to-mesenchymal transition

GRP-78-Bip= glucose-regulated protein 78/ binding immunoglobullin protein

RISC= RNA-induced silencing complex

Ago 2= Argonatute protein 2

MREs= miRNA response element

Introduction

Metastasis, which accounts for 90% of all cancer-related mortality (1), consists of a series of consecutive biological steps to allow secondary tumor formation through initial invading basement membrane, entering the circulation through lymphatic channels or blood vessels, adopting new environment and proliferating to form secondary tumor. Among these processes, cell migration is a critical determinant of early stage cancer metastasis by contributing to enhance cancer invasion. Cell migration is highly associated with integrated dynamics of cytoskeleton and adhesions molecules (2). These cells can adhere to extracellular matrix to obtain abilities to migrate, to survive and to transmit signal. Hence, cell migration has been found association with wound repair, cell differentiation, embryonic development, epithelial -to-mesenchymal transition (EMT) of cancer cell, and metastasis of tumor (3). Therefore, better understanding genes involved in cancer cell migration hold great promise for developing inhibitors aimed at preventing cancer metastasis.

We and others have recently demonstrated that Cell Migration Inducing Protein (CEMIP) plays a critical role in cancer cell proliferation and invasion. CEMIP has been found to serve as a novel biomarker due to its expression inverse correlation with patient survival rate (4). CEMIP or KIAA1199 consists of 30 exons and the 28 exons are coding exons for CEMIP gene expression. The exon 30 encodes 3'-untranslated region of CEMIP (KIAA1199) that plays role for the stability of mRNA of CEMIP (5). It has been reported that genetic mutations of CEMIP (KIAA1199) associates with non-syndromic hearing loss, and hence the CEMIP gene appears to be essential for normal auditory function (6). Emerging evidence has suggested that CEMIP plays critical role in cancers, including breast cancer, gastric cancer, and colon cancer. We previously unraveled the molecular mechanism underlying CEMIP-mediated cancer cell migration (REF). We demonstrated that CEMIP is a novel endoplasmic reticulum (ER) resident protein through its interaction with GRP-78 (Bip). This interaction results in ER calcium release, leading to a cascade of signaling transduction via the activation of protein kinase C alpha (PKCa) and its downstream effectors which causes enhanced cell migration (4).

CEMIP gene has been found to be upregulated in various human cancers, however, its regulatory mechanism is just beginning to be understood. It has been reported that CEMIP promoter is tightly regulated by AP-1 and NF-kB. Identification of CEMIP promoter demethylation provides additional regulatory mechanism of CEMIP expression (7). However, the role of the 3' UTR in the regulation of CEMIP expression remains to be characterized, specifically for post-transcriptionally regulation of CEMIP expression by a small non-coding microRNA.

MicroRNA (miRNA), 19-25 nucleotides in length, are short noncoding RNAs typically encoded within introns. It was first discovered in 1993, when the miRNA lin-4 was found to downregulate expression of the gene lin-14 in *Caenorhabditis elegans* (8-9), and the discovery of the miRNA let-7 in 2000 (10-11), with homologs in other species including metazoans, plants and viruses, as well as a few in protists and slime mold, and human. Accumulated evidence had showed that miRNA genes were frequently located near cancer-associated genomic regions (12). It has been demonstrated that miRNAs regulate the expression of their target genes related to tumorigenesis and metastasis, including cell cycle regulation, differentiation, apoptosis and invasion (13).

In eukaryotic cells, miRNA are first transcribed as a long RNA transcript to generate primary miRNA (pri-miRNA) with an imperfectly base-paired hairpin structure (14). These pri-miRNA is cleaved by RNasesIII enzyme Drosha within nucleus to form a short stem loop precursor miRNA (pre-miRNA). Next, the pre-miRNA is exported from the nucleus by Ran-GTP dependent Exportin-5, and cleaved in cytoplasm by another RNasesIII type enzyme Dicer, into mature microRNAs. These mature miRNAs are now loaded onto the RNA-induced silencing complex (RISC), in which a guide protein called Argonatute protein 2 (Ago2) within the RISC targets to mRNA by the miRNA strand, typically base pairs in the 3' untranslated region of the mRNA, signaling the target for translational repression. So far more than 1500 miRNAs have been found in humans is proposed to regulate about 30% of human genes (15). The biogenesis and function of miRNAs is highly regulated where the regulations takes place at multiple steps including their transcription, their processing, and their loading onto AGO proteins and miRNA turnover, and their dysfunction often causes with human disease, such as cancer and neurodevelopmental disorder (16).

The miRNA-128 family encoded by two distinct genes, miR-128-1 and miR-128-2, which are located in the introns of R3HDM1 and RCS (ARPP-21, cyclicAMP-regulated phosphoprotein) genes within human chromosome 2q21.3 and 3p22.3 respectively (17). However, the two distinct genes generate the same mature miRNAs with identical sequence, miR-128. It has shown about 26% of the mammalian intronic miRNAs might be transcribed from their own promoters (18), and in particular miR-128 had Pol III promoter in its 5′-flanking region, which might permit an independent expression of its host gene ARPP-21 (19).

In this study, we first identify miRNA-128 directly targets to the 3'UTR of CEMIP mRNA to repress its translation, resulting in decreased cell migration. As for regulation of miR-128-1, we found overexpression of Snail-1, a transcription factor attenuates miR-128-1 expression. Together, our study demonstrates the role of miR-128 in cancer dissemination, and unravels a possible molecular target for inhibition of cancer metastasis.

Material and Methods

Cell Lines and Transient Transfections

All cell lines were purchased from ATCC (Manassas, VA). COS- 1 monkey kidney epithelial, human fibrosarcoma HT1080 and NIH3T3 cell line, and MCF-7 and MDA-MB 231 human breast cancer cell lines were maintained in the DMEM (Invitrogen) containing 10% FBS. Transfection of plasmid DNA into cells was achieved using polyethylenimine (Polysciences) and the transfected cells were incubated for 48 h at 37C followed by biochemical and biological assays.

Quantitative Real-Time PCR

RNA from cells was isolated using Qiagen RN easy Kit according to the manufacturer's instructions. RNA was reverse transcribed to generate cDNA using Reverse Transcriptase (Bio Rad iScript cDNA Synthesis Kit). Quantitative real-time PCR was performed using BioRad IQ SYBR-Green Super Mix on a BioRad iQ5 Real Time PCR machine. Relative expression was calculated using the DDCt method. HPRT-1 and GAPDH were used as internal controls.

DNA constructs

2.0 vector that harbors GFP as a reporter protein (Addgene). We amplified miR-128-1 that includes the approximately 22-nt mature miRNA-128-1 and 100 nt of genomic sequence flanking each side of the miR-128-1 from human genomic DNAs extracted from human umbilical vein endothelial cells (HUVEC). Based on miR-128-1 in chromosome 2, we designed primers as follows: forward primer #2921: 5'ATCTCGAGCTAGCTGTTTTCTGTGTAGC3'; and reverse primer #2922: 5'ATGAATTCA GGTATTACAATTAATGAAA3'). The PCR fragment containing miRNA128-1 was then cloned into MDH1-PGK-GFP 2.0 vector at XhoI and EcoRI sites to generate the miRNA-128-1/GFP construct.

To study the effect of miRNA-128 on CEMIP expression via the 3'UTR, a reporter gene system was generated. pGL3 promoter-vector (Promega) containing firefly luciferase driven by a pSV40 promoter was employed. A 3.3 kb DNA fragment of CEMIP 3'UTR was amplified by PCR using CEMIP/3'UTR as a template (forward primer #2962: 5'ATCTAGA GGACAGCTGCCGCCCGGTGC-3'3'; and reverse primer #2963: 5'-GGGTTAAC GATGATGACATCCTTTGTATGTTTACTATAATAA-3') and cloned into pGL3 promoter-vector at XbaI site to generate Luciferase/3'UTR (Luc/3'UTR). To determine the specificity of the

miRNA-128 response element within the 3'UTR, a site direct mutagenesis approach was employed to convert the core miRNA-128 binding site (TGA CAC) to complementary nucleotides (GTG TCA) in the Luc/3'UTR plasmid DNA (Luc/3'UTR^{mu}).

To generate an inhibitor of miR-128-1 for downregulation of miR-128-1 in less invasive, high miR-128-1 expressing cancer cells, we generated a miR-128-1 sponge based on a published report for miRNA sponge design (4). The miR-128-1sponge contains: 1) 22 nt mismatched antisense of miR-128-1 in each repeat (mature miR-128-1: 23 nt) ; 2) a bulge area with a nt at position 9 missing and position 10-12 mismatched from the 5' end of miR-128-1 mature sequence; 3) four tandem repeats of the miR-128-1 binding site with a 4 nt spacer sequence (AATT) between the repeats; 4) 5' and 3' caps; and 5) a poly-A tail at the 3' end. The first half of the miR-128-1 sponge was generated by annealing sense and anti-sense oligos (sense oligo_3136: 5' GATCCGACGGCGCAAGGATCATC AACATCACAGTGTGAGGCTCTTTAATTTCACAGTGTGAGGT CTC TTT G 3'; and antisense_3137: 5' GCTGCCGCGATCCTAGTAGTTGT AGTGTCACACTCCAGAGAAATTAAAGTGTCACACTCCAGAGAAACTTAA 3') containing BamHI and EcoRI sites. The double stranded DNA insert was then cloned into pSIREN/RetroQ vector (Clontech). The resultant construct then received the second half of the miR-128-1 sponge with annealed doubled strand oligos (sense_3138: 5'[phos] AATTCTCACAGTGTGGGTCTCTTTAGATGATCCTAGCGCCGTCTTTTT G 3'; and antisense_3139: 55'[phos] AATTCAAAGACGGCGCTAGGATCATCTAAAGAGACCTCACACTGTGAAATTAAAGAG ACCTCACACTGTGAG 3' at the EcoRI site to generate the full length sponge construct.

All constructs were confirmed by DNA sequencing.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue sections (FFPE) tissue sections (5 μm) of human adult breast tissue containing either benign or breast carcinoma tissues, as well as sections of human colon cancer specimens from Stony Brook University Research Histology Core Lab approved by the Institutional Review Boards of Stony Brook University were examined by a modified IHC method (26). Antigen retrieval was achieved by boiling tissue sections for 30 minutes in 0.01M sodium citrate, pH 4.

Sections were blocked for one hour in 1% BSA at room temperature and incubated in rabbit anti-CEMIP antibodies, at 1:250 dilution at 4°C overnight. After washing, samples were incubated

with HRP-conjugated anti-rabbit IgG at 1:100 dilution, and then by Biotin-XX-Tyramide amplification (Invitrogen, Carlsbad, CA), performed according to manufacturer's instructions, and streptavidin-HRP at 1:100 dilution. Stained sections were visualized using 3,3'-diaminebenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. IHC staining without primary antibody was used as a negative control.

Results

Upregulated expression of CEMIP in aggressive human cancer

To determine the clinical relevance of CEMIP expression in cancer progression, colon cancer specimens were examined for the expression of CEMIP by examining mRNA and protein levels. In order to determine whether CEMIP expression occurs in cancer cells or stromal cells, a laser capture microdissection (LCM) technique was employed to harvest colon carcinoma cells and tumor adjacent normal epithelial cells followed by real-time RT-PCR for the mRNA of CEMIP. Our data shows that CEMIP is selectively expressed in human colon carcinoma and in invasive colon cancer cells, but not in normal epithelial cells. Expression of CEMIP in invasive cancer cells is elevated as compared to non-invasive tumor cells, although there is no statistical significance among them (Figure 1A).

To validate this observation, human colon cancer specimens were examined by IHC using the anti-CEMIP antibody. As we expected, minimal expression of CEMIP is detected in cancer adjacent normal cells, but increased intensity of staining is found in cancer cells at the invasive front (Figure 1B). Thus, CEMIP is upregulated in human cancers and the expression level of CEMIP is correlated with the invasive status of human cancer. Theses results suggest the function of CEMIP in cancer progression.

Identification of miR-128-1 response element within the 3'UTR of CEMIP by a computational analysis

CEMIP is overexpressed in human cancers, resulting in enhanced cell migration and poor patient outcome(4). However, the regulatory mechanism of CEMIP in human cancer is not fully understood. It has been reported that 3' untranslated regions of mRNAs often contains miRNA response element (MREs). We then analyzed the 3'UTR of CEMIP mRNA for predicted miRNA response elements using a web-based program, TargetScan. We identified a potential miR-128-1 MRE within the 3'UTR of CEMIP between 2256-2263 nucleotides (Figure 2A). To confirm the bioinformatics data, RNAs from a panel of cancer cell lines were extracted followed by qPCR analysis using miR-128-1 primers. A high level of miR-128-1 was found in less invasive cancer cell lines, such as MCF-7 as well as non-cancer NIH3T3 fibroblast cells. In contrast, minimal expression of miR-181 was identified in highly invasive cancer cells, e.g. HT1080, MDA-MB-231 (Figure 2B, left).Interestingly, CEMIP were found negatively correlated to miR-128-1 expression as examined by real time RT- PCR(Figure 2B, right) and Western blotting (HT1080 and MDA-MB-231, Figure 2D). Our data suggest that CEMIP may be regulated by miRNA 128-1.

To further determine the clinical significance of miR128 in regulation of CEMIP, we

examined the expression of miR-128-1 in human cancer specimens. We harvested tumor cells at the invasive front of human colon cancer specimens as well as tumor adjacent normal epithelial cells using a microdissection technique followed by real-time RT-PCR for miR-128-1. Consistently, miR-128-1 is downregulated in invasive tumor cells as compared to tumor adjacent normal epithelial cells (Figure 2C). Together, these data suggest that expression of CEMIP in human invasive cancer is affected by miR-128-1 levels.

Interference of ectopically expressed CEMIP by miR-128

To identify the ability of miR-128 in regulation of CEMIP expression, we generated a retroviral construct containing for expressing miR-128 using MDH1-PGK-GFP 2.0 vector. HT1080 cells that express low level of miR-128 and high level of CEMIP were infected by retrovirus encoding miR128. Retrovirus containing scrambled sequence was used as a control. In Figure 3A, it confirms the transient transfection efficiency as showing higher miR-128-1 expression, but not in miR-control. Ectopic expression of miR-128, but not miR-control significantly decreases CEMIP mRNA level examined by qualitatively real-time RT-PCR (Figure 3B). Furthermore, ectopic expression of miR-128 decreased CEMIP protein expression by Western blotting using an anti-CEMIP antibody in overexpressed miR-128-1 HT1080 cells (Figure 3C, left). In addition, to determine whether the additional bands (located below and above the molecular weight of CEMIP shown on Fig. 3C) are CEMIP isoform or degradation products with respect to CEMIP expression, we introduced CEMIP small hairpin RNA (ShRNA) to knockdown CEMIP. Further western blot analysis suggested those band do not relate with CEMIP protein as they were not silenced when shCEMIP were expressed (Figure 3C, right).

miR-128-1 directly targets CEMIP at 3'UTR

To determine weather miR-128 directly interacts with CEMIP 3'UTR, we generated a firefly luciferase reporter gene, in which the 3'UTR of CEMIP with miR-128-1 predicted binging sites was fused to the C-terminus of luciferase reporter gene. Renilla report gene that serves as a normalization control, was co-transfected with miR-control or miR-128-1/GFP, and 3'UTR of CEMIP luciferase gene. We also engineered a deletion mutant by scrambling the MRE sequence of miR128 within the 3'UTR of CEMIP (Luc/3' UTR mu). Expression of miR-128-1 along with the reporter gene displayed lower levels of luciferase activity as compared to the miR-control (Figure. 4A). When the MRE was mutated, miR128 no long affect the reporter gene activity. Thus, it suggests that the downregulation of CEMIP by miR-128 is thorugh directly targeting to the single cognate recognition site within 3'UTR of CEMIP mRNA.

To further validate the role of miR-128-1 in regulation of CEMIP 3'UTR expression, a U6 promoter-driven miR-128-1 inhibitor (sponge) was generated to inhibit miR-128-1 expression

(Figure 4B). When the sponges were stably expressed into MCF-7 cells, which is detected a high level of endogenous miR-128-1, the miR-128-1 level is significantly diminished in the cells expressing the sponge as compared to the control cells (Figure 4C). When Luc/3'UTR reporter gene was transfected into MCF-7 cells stably expressing miR-128-1 sponges, the luciferase activity increased in comparison to miR-control sponge infected cells, whereas luciferase activity from Luc/3'UTR^{mu} was not affected by miR-128-1 sponge (Figure 4D) .

To further determine whether the decreased CEMIP expression in both mRNA and protein levels by miR-128-1 is due to the existence of the 3'UTR of CEMIP, we generated plasmid DNA encoding only the open reading frame of CEMIP, which lacks the 3'UTR of CEMIP as well as a construct encoding both ORF and 3'UTR. The plasmid of CEMIP 3'UTR was co-transfected with either miR-128-1 or miR-control. Western blot indicated decreased CEMIP mRNA level in miR-128/C43/3'UTR expressing vector (Figure 4E). But, there was no distinct difference in ectopic CEMIP expression between the cells that overexpressed either miR-128-1 or miR-control (Figure 4F). Therefore, these data further support that miR-128-1 is directly target to CEMIP 3'UTR.

Decrease CEMIP-induced migration by miR-128-1

Our lab previously demonstrated that expression of CEMIP in MCF-7 (less aggressive cancer cells) induces epithelial-to-mesenchymal transition (4), which is accomplished with increased cell migration. Conversely, silencing CEMIP in highly invasive MDA-MB-231 cells results in MET conversion with decreased cell migration. (7). We then examine if reduced CEMIP expression by miR-128 leads to decreased cell migration by using transwell chamber migration assay. HT1080, which is a invasively migratory fibrosarcoma cells that highly expresses CEMIP. Interestingly, transiently transfected with miR-128 significantly decrease cell migratory ability, as compared to miR-control transfected cells (Figure 5A). This finding suggests miR-128 is essential to regulate cell migratory ability through affecting CEMIP expression.

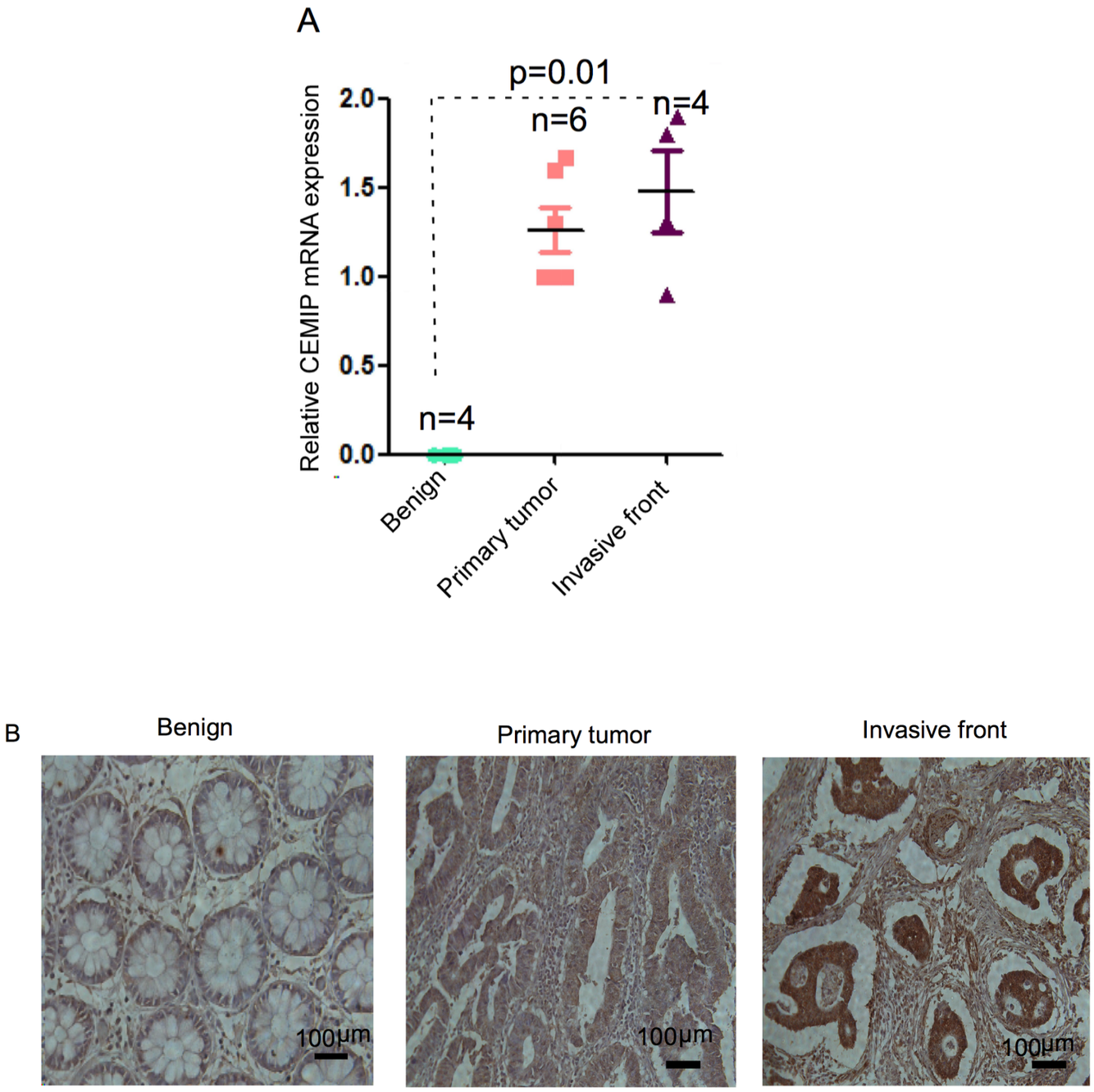

Figure 1. **Upregulation of CEMIP in invasive human cancers.** **(A)** Analysis of CEMIP mRNA expression using real-time PCR for total RNA isolated from archived FFPE tissue sections of primary tumor, invasive front and adjacent normal tissue from colon cancer using a LCM technique. **(B)** Examination of CEMIP expression in tumor adjacent normal tissue, adenocarcinoma, and invasive adenocarcinoma of the colon by IHC.

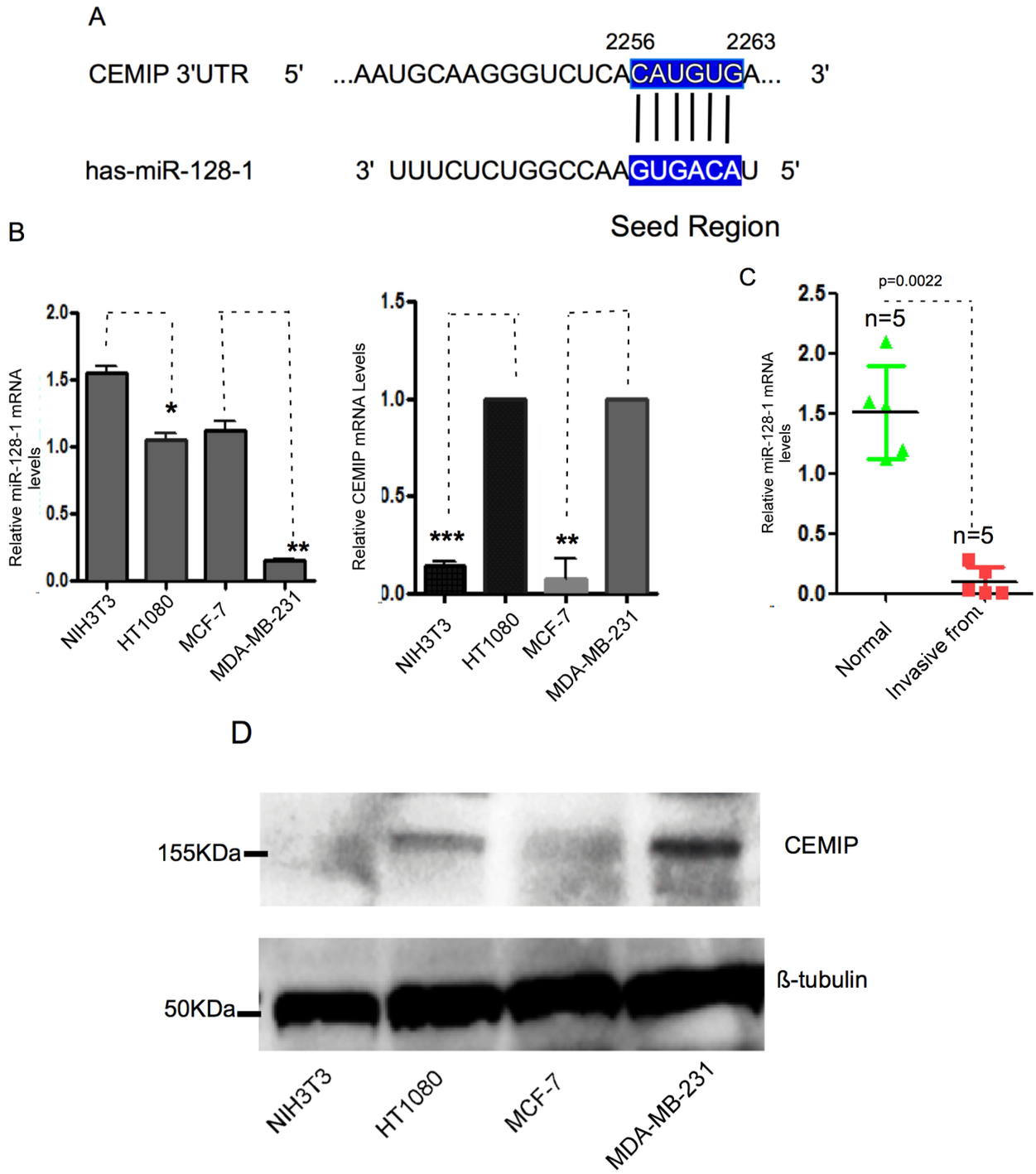

Figure 2. Inverse correlation between miR-128-1 and CEMIP in human cancer cells. (A) Computational analysis of the miRNA response element within the CEMIP 3'UTR predicted a miR-128-1 binding site. **(B)** Increased CEMIP expression, but reduced miR-128-1 expression in invasive cancer cell lines by real-time PCR, and **(C)** miR-128-1 levels in tumor adjacent normal cells and tumor cells at the invasive front isolated from FFPE sections by microdissection technique. **(D)** Western blotting using an anti-CEMIP antibody.

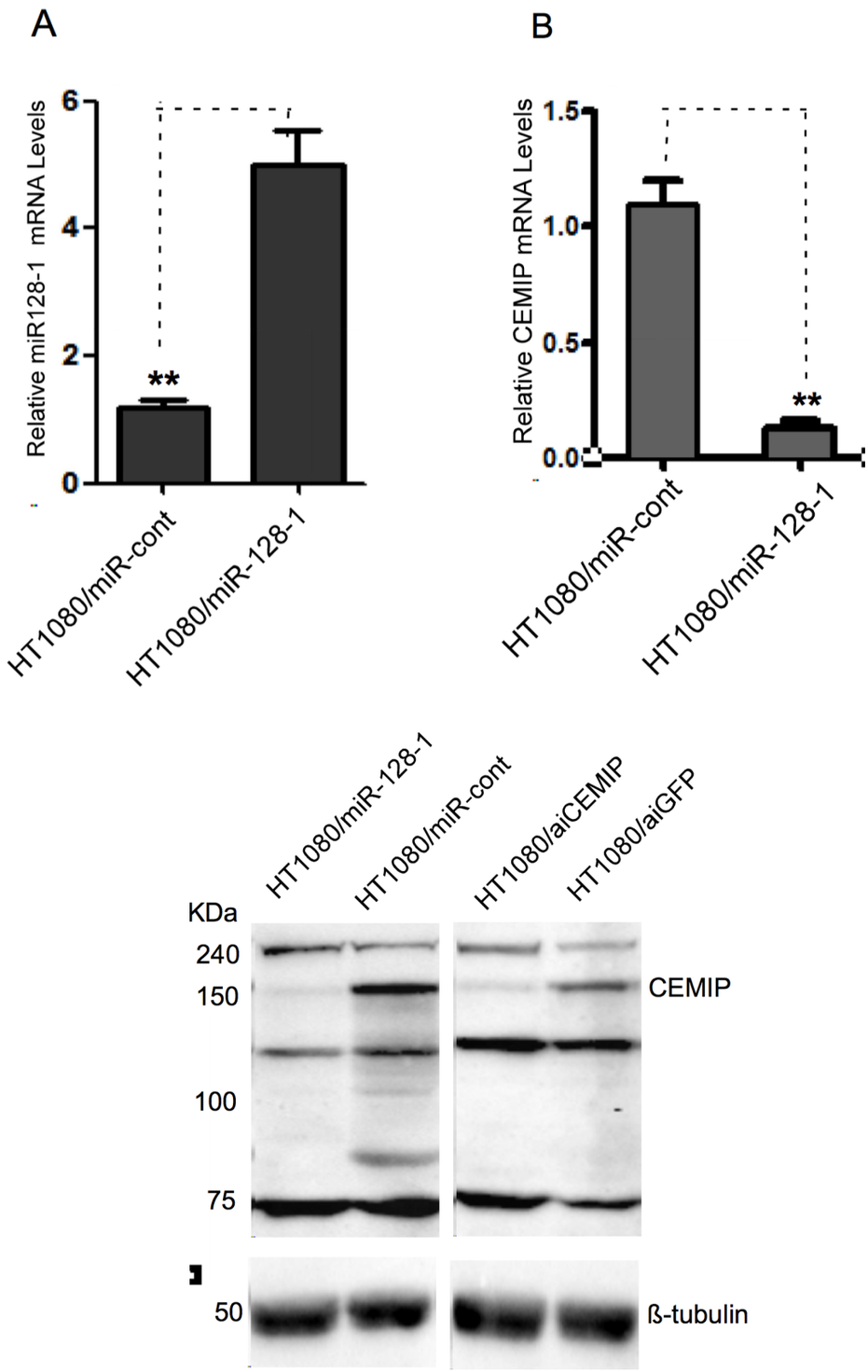

Figure 3. Interference with ectopically expressed CEMIP by miR-128-1. (A) Expression of miR-128-1, and **(B)** CEMIP in transiently expressing the corresponding miR-128-1 and miR-control by real-time RT-PCR. Levels of the expression were normalized by HPRT-1. **(C)** Total lystates isolated from HT1080 cells expressing cDNA as indicated were examined for CEMIP using anti-CEMIP antibody (tubulin as a loading control), and introducing shCEMIP cDNA into HT1080 cells indicated denatured non-specific proteins on the blot.

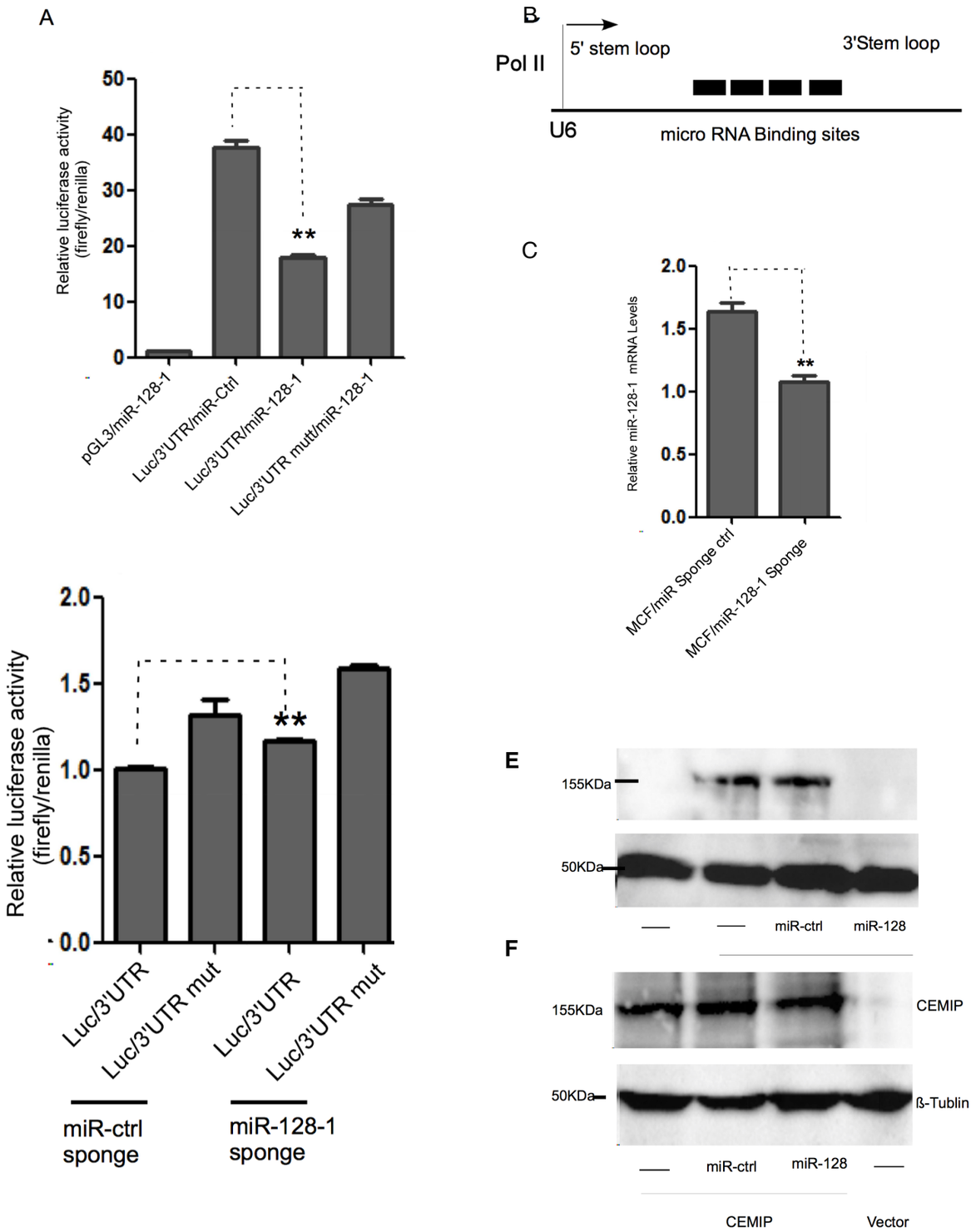

Figure 4. Direct interaction of miR-128-1 with the CEMIP 3'UTR. (A) A reporter gene assay was performing in HT1080 cells transfected with cDNAs as indicated.**(B)** A shame diagram of miR-128-1 sponge. **(C)** MCF-7 cells stably expressing sponges as indicated were examined for miR-128-1 expression by RT-PCR. **(D)** MCF-7 cells stably expressing the sponges as indicated were transfected with reporter genes and followed by a dual luciferase assay. **(E)** RT-PCR indicated decreased CEMIP mRNA level in miR-128/C43/3'UTR expressing vector as well as decrease CEMIP protein level, but **(F)** no effect on CEMIP expression by miR-128-1 in HT1080 cells transfected with cDNA encoding CEMIP without the 3'UTR as examined by real-time RT-PCR Western blotting.

A

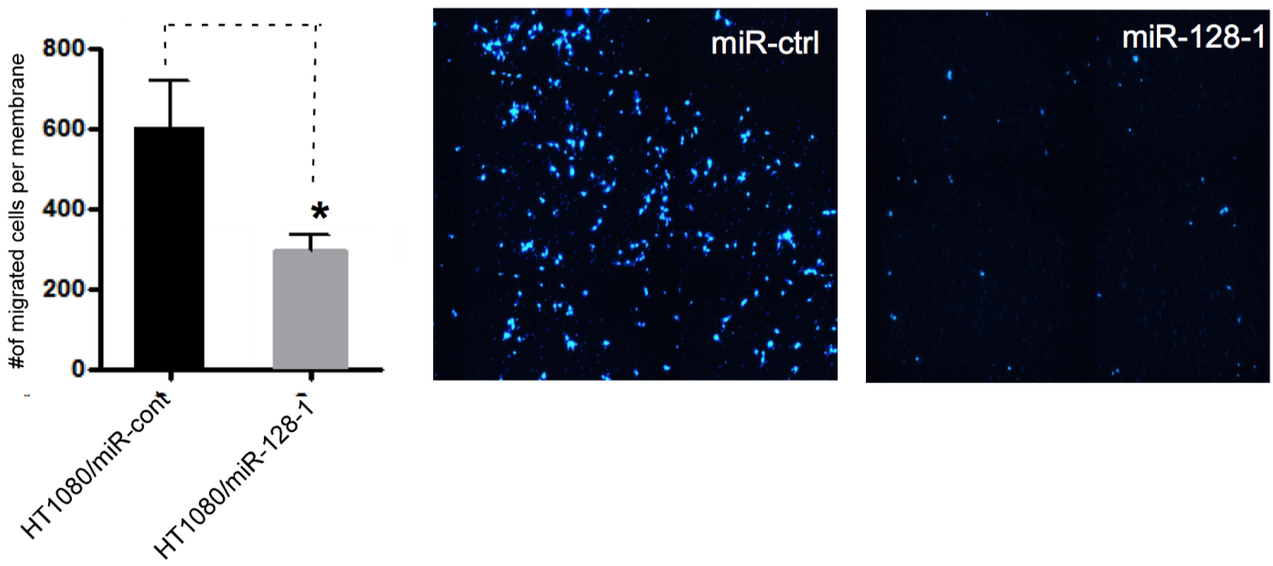

Figure 5. Overexpression of miR-128-1 attenuates CEMIP-induced cell migration activity. (A) transwell chamber migration assay was performed with HT-1080 cells stably expressing miR-128-1 and miR-control. Migrated cells were automatically counted based on nuclear staining. Representative images are presented.

Discussion

In this study, we first validated the clinical relevance that CEMIP is highly expressed in human colon cancers, which has also confirmed our previously published data. We then demonstrated that miR-128-1 is inversely correlated with CEMIP expression and the invasive capacity of cancer cell lines. We also identified the miR-128-1 target sequence within the CEMIP 3'UTR that is responsible for the stability of CEMIP mRNA. Ectopic expression of miR-128-1 resulted in downregulation of both endogenous and exogenous CEMIP expression, leading to decreased CEMIP-mediated cell migration (4). Previously, we have demonstrated CEMIP is transcriptionally regulated by activating its promoter through AP-1 and NF-kB (7), the effect of the stability of CEMIP mRNA is another key regulatory mechanism in controlling CEMIP expression. Therefore, our observations identified the post-transcriptional regulatory mechanism for CEMIP expression by miR-128-1.

Our data indicated that miR-128-1 negatively affects CEMIP expression through binding to the 3'UTR of CEMIP. Since upregulation of CEMIP directly associates with cancer aggressiveness (4), induction of endogenous miR-128-1 provides a potential approach to prevent cancer invasion and metastasis. However, it should be mentioned that the role of miR-128-1 in cancer is still controversial depending on the tumor type. In hematopoietic malignant tumors, it has been measured the levels of miR-128 expression in the blood of 147 newly diagnosed acute leukemia patients and found that miR-128 was highly expressed in acute lymphoblastic leukemia (ALL) patients (20). On the other hand, miR-128-1 was upregulated in undifferentiated gastric cancer tissues while miR-128-2 was downregulated in the specimens of 42 undifferentiated gastric cancer tissues and paired controls (21). Therefore, miR-128 has been suggested its dual role in tumor enhancer and tumor suppressor under different types of human cancer. These controversial observations suggest the complexity of miRNAs and the function of specific miRNAs can differ markedly depending on tumor types. In our study, we showed that miR-128-1 and CEMIP are differentially expressed in invasive tumor cells and tumor adjacent normal cells.

In this study, we also studied the molecular mechanism underlying how miR-128-1 expression is regulated. It has shown about 26% of the mammalian intronic miRNAs including miR-128-1 might be transcribed from their own promoters (18). It is possible that miR-128-1 expression can be regulated by transcription factors. Based on Qian's study, they searched conserved transcription factor binding sites for miR-128 by use of rVista 2.0 (22). They identified 2 conserved E- box motifs, relative to the transcription start site of the human miR-128, and found SNAIL family (SNAIL, SLUG, and SMUC), ZEB (ZEB1 and ZEB2) have been reported to bind to E-box sequences (CANNTG) present in the promoters of genes (23).

Hence, we searched conserved miR-128-1 promoter region using of PROmiRNA (24), and identified three conserved promoter regions, which are also contained snail-1 binding motif. To further identify ability of Snail-1 in regulation of miR-128-1 expression, we examined whether snail-1 can affect ectopically expression of miR-128-1. Ectopic expression of snail-1 but not GFP control significantly decrease miR-128-1 level but increase CEMIP level examined by qualitatively real-time RT-PCR (Figure 6A).

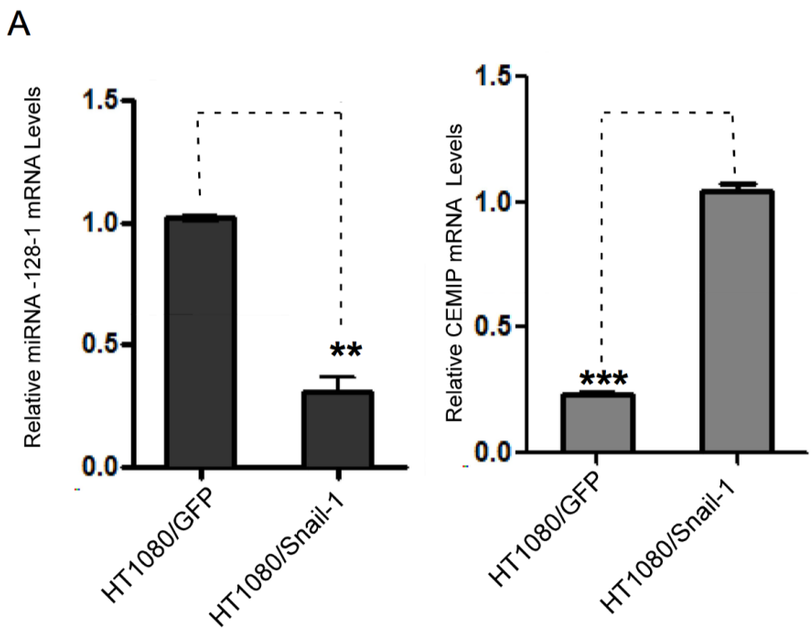

Figure 6. A transcription factor, snail-1 regulated miR-128-1 expression. (A) stably expressing cDNA as indicated, increased CEMIP expression, but reduced miR-128-1 expression in invasive cancer cell lines by real-time PCR.

Although further analysis need to be preformed to confirm whether snail-1 potentially regulated miR-128-1 expression, our preliminary data provide a clue of how miR-128-1 is regulated. Mechanistically, our data showed miR-128-1 is downregulated by the transcription factor snail-1 by directly or indirectly binding to its promoter region. Ectopic expression of Snail-1 reduced endogenous miR-128-1 expression and therefore induced CEMIP expression. Further experiment such as ChIP assay can be performed to specify the promoter region of miR-128-1 among the three conserved regions from PROmiRNA prediction. Since it has predicted snail-1 binding sites on these conserved promoters, an anti-snail-1 antibody can be used to pull down, and followed by qPCR analysis using primers that identify prompters.

In conclusion, the current report provides evidence inducing endogenous miR-128-1 expression to reduce CEMIP-mediated cancer cell migration. However, miR-128-1 can potentially target over 600 different mRNAs by PicTar prediction algorithm. These miR-128-1targeted genes are differential or have effects in different cellular contexts. Thereby, the challenge ahead is to resolve inconsistent observations and unify the current data into a coherent mechanism for miR-128-1 function within particular tumor types. Interestingly, recent studies using a DNA microarray or quantitative mass spectrometry approach indicate that the repression of mRNAs and proteins by a miRNA is often relatively small (less than 2 fold and rarely exceeds 4 fold) (25,26). Given the technical limitation, the only way to validate the effects of reduced gene expression by a miRNA on cellular phenotypic changes is to characterize the miRNA-targeted gene individually. Nevertheless, our findings identify an additional post-transcriptional mechanism, a novel regulatory pathway in invasive cancer cells, in which the upregulated transcription factor, Snail-1, reduces the expression of miR128-1, leading to stabilizing CEMIP mRNA, so expressed CEMIP then induce cancer cell migration.

Reference

1. Fidler, I. J. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nature Rev. Cancer* **3,** 453–458 (2003).

2. Taro Toyota, Yuichi Wakamoto, Kumiko Hayashi and Kiyoshi Ohnuma (2012). Controlling Cell Migration with Micropatterns, Innovations in Biotechnology, Dr. Eddy C. Agbo (Ed.), ISBN: 978-953-51-0096-6, InTech, Available from: http://www.intechopen.com/books/innovations-in-biotechnology/controlling-cell-migration-with- micropatterns.

3. Thiery, J. P. Epithelial–mesenchymal transitions in tumour progression. *Nature Rev. Cancer* **2,** 442–454 (2002).

4. Evensen Nikki, Kuscu Cem, et al., Unraveling the Role of KIAA1199, a Novel Endoplasmic Reticulum Protein, in Cancer Cell Migration. Journal of National Cancer Institute. September 18,,2013,,Volume15, Number 18, 1402-1416.

5. Nakayama M, Kikuno R, Ohara O (2002) Protein-protein interactions between large proteins: two-hybrid screening using a functionally classified library composed of long cDNAs. Genome Res 12: 1773–1784.

6. Abe S, Usami S, Nakamura Y (2003) Mutations in the gene encoding KIAA1199 protein, an inner-ear protein expressed in Deiters' cells and the fibrocytes, as the cause of nonsyndromic hearing loss. J Hum Genet 48: 564– 570.

7. Kuscu C, Evensen N, Kim D, Hu Y-J, Zucker S, et al. (2012) Transcriptional and Epigenetic Regulation of KIAA1199 Gene Expression in Human Breast Cancer. PLoS ONE 7(9): e44661. doi:10.1371/journal.pone.0044661.

8. Wightman B, Ha I, Ruvkun G, 1993, Cell. 75:855-862.

9. Lee RC, Feinbaum RL, Ambros V, 1993, Cell. 75:843-854.

10. SlackFJ,BassonM,LiuZ,AmbrosV,HorvitzHR,RuvkunG,2000,MolCell.5:659-669.

11. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G, 2000, Nature. 403:901-906.

12. P. Lamy, C.L. Andersen, L. Dyrskjøt, et al., Are microRNAs located in genomic regions associated with cancer?, Br. J. Cancer 95 (10) (2006) 1415–1418.

13. T.A. Farazi, J.I. Spitzer, P. Morozov, et al., miRNAs in human cancer, J. Pathol. 223 (2) (2011) 102–115.

14. Bushati N, Cohen SM, 2007, Annu Rev Cell Dev Biol. 23:175-205.

15. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic acids research 2006;34(Database issue):D140-4.

16. Ha, Minju, V. Narry Kim, Regulation of microRNA biogenesis. Nature Reviews Molecular Cell Biology. 2014, Vol 15.August 2014.

17. I.G. Bruno, R. Karam, L. Huang, et al., Identification of a microRNA that activates gene expression by repressing nonsense- mediated RNA decay, Mol. Cell 42(4) (2011) 500-510.

18. D.L. Corcoran, K.V. Pandit, B. Gordon, et al., Features of mammalian micro RNA promoters emerge from polymerase II chromatin immunoprecipitation data, PLoS One4 (4)(2009) e 5279.

19. A.M. Monteys, R.M. Spengler, J. Wan, et al., Structure and activity of putative intronic miRNA promoters, RNA 16 (3) (2010) 495–505.

20. Y.D. Zhu, L. Wang, C. Sun, et al., Distinctive microRNA signature is associated with the diagnosis and prognosis of acute leukemia, Med. Oncol. 29 (4) (2011) 2323–2331.

21. T. Katada, H. Ishiguro, Y. Kuwabara, et al., microRNA expression profile in undifferentiated gastric cancer, Int. J. Oncol. 34 (2) (2009) 537–542.

22. Loots GG, Ovcharenko I. rVISTA 2.0: evolutionary analysis of transcription factor binding sites. Nucl Acids Res 2004; 32: W217–21.

23. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 2007;7:415–28.

24. Annalisa Marsico.Matthew R Huska, et al., PROmiRNA: a new miRNA promoter recognition method uncovers the complex regulation of intronic miRNAs. Genome Biology 2013, 14:R84 doi:10.1186/gb-2013-14-8-r84.

25. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature 2008;455(7209):64-71.

26. Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature 2008;455(7209):58-63.