miR-106b modulates cancer stem cell characteristics through TGF-β/Smad signaling pathway in CD44 positive gastric cancer cells

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Directed by Professor Yong Chan Lee

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This certifies that the Master's Thesis of Dayeon Yu is approved.

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<TABLE OF CONTENTS>

<LIST OF FIGURES>

<LIST OF TABLES>

<ABSTRACT>

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Cancer stem cells have the capacity to form new tumors and are thus considered to be a cause of metastasis and tumor recurrence. However, many of the mechanisms determining cancer stem cell characteristics are still unknown. MicroRNAs (miRNAs) are studied as possible modulator of cancer stem cell generation and the retention of cancer stem cell characteristics.

The aim of this study was to examine the miRNA expression profile regulating the cancer stem cell characteristics of CD44 positive gastric cancer cells. We sorted gastric cancer stem-like cells using the cell surface stem cell marker, CD44 using fluorescence-activated cell sorting (FACS). CD44(+) cells formed more and larger spheres than CD44(-) cells. Cancer stemness related markers; *Oct4*, *Bmi*, *Nestin*, and *ABCG2* were overexpressed in CD44(+) cells at the mRNA and protein levels. CD44(+) cells showed increased tendency for increased expression of mesenchymal markers while epithelial cell markers were downregulated. In miRNA

microarray, the miR-106b family; miR-106b, miR-93, and miR-25 was significantly upregulated in CD44(+) cells compare to CD44(-) cells. Smad7, which inhibits TGF-β/Smad signaling as a target of the miR-106b family, was downregulated in CD44(+) cells. Furthermore, expression of TGF-β/Smad signal molecules were activated in CD44(+) cells, in accordance with the action of miR-106b family. Inhibition of miR-106b showed suppression of TGF-β/Smad signaling pathway and decreasing of cell invasiveness. Our study suggested that CD44(+) gastric cancer cells showed cancer stem cell properties with epithelial-mesenchymal transition (EMT) characteristics. miR-106b family regulated cancer stem cell properties, particularly EMT characteristics, through the TGF-β/Smad signaling pathway. Taken together, these results indicate that targeting miR-106b might be an effective cancer therapy in gastric cancer through the modulation of cancer stem cell characteristics.

Key words : gastric cancer, stem cell, miRNA, epithelial-mesenchymal transition

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I. INTRODUCTION

Gastric cancer has the second highest mortality rate among cancers and is the fourth most common cancer in the world with 738,000 deaths and 989,000 new cases in 2008 [1]. *H. pylori* infection and dietary factors are thought to be the main causes of this phenomenon [2]. Cancer stem cells are known tumor initiating cells, and display unique characteristics that differentiate them from other cancer cells. Like all stem cells, cancer stem cells have self-renewal properties, the potential to differentiate into any cells in the tumor population and a considerable proliferative capacity [3]. Also, cancer stem cells are considered to be major causes of tumor metastasis and reccurence due to their mesenchymal characteristics.

Cancer stem cells have been identified and isolated in breast, brain, and several other cancers since originally being discovered in the hematopoietic system [4-6]. Several studies have also recently demonstrated the existence of cancer stem cells in gastric cancer [7,8]. One of the well-known techniques to

fractionate cancer stem cells employs several surface markers overexpressed in cancer stem cells. CD44 is a transmembrane glycoprotein widely used to isolate cancer stem cells in various cancers, particularly in gastric cancer [9].

Epithelial-mesenchymal transition (EMT) play important role in embryonic development in many animals. Epithelial cells have polarity and are tightly connected to each other through cell junctions, while mesenchymal cells are highly mobile. In the EMT process, epithelial cells lose their characteristics and then take on mesenchymal characteristics. EMT is considered a target of cancer therapy because it confers on cancer cells invasiveness and metastasis [10]. In addition, EMT shares several properties with cancer stem cells. A study showed that fraction of $CD44^{high}/CD24^{low}$ cancer stem-like cells are increased when EMT was induced in mammary epithelial cells [11].

MicroRNA, a small non-coding RNA (~22 nt), has drawn attention in recent years as one of the key modulators of cellular properties. An miRNA network is a complicated system and its dynamic interaction with various targets regulates many cellular processes by altering gene expressions through cleavage of target mRNA, translational repression and mRNA deadenylation [12]. Also, miRNA may be an important regulator of cancer stem cell properties. miR-200c suppresses cell differentiation by modulation of bmi1 in breast cancer cells [13] and miR-34a represses generation of prostate cancer stem cells [14]. The miR-106b family is overexpressed in several tumors, including those of gastric cancer [15-18]. miR-106b family is implicated in many cellular signaling pathways; regulation of cell cycle through p21 [19] and activation of TGF-β signaling pathway [20].

We have fractionated the gastric cancer cells by CD44 expression and identified cancer stem cells phenotypes in CD44(+) cells. We then evaluated whether there were any differences in miRNA expressions between CD44(+) and CD44(-) cells to find candidate miRNA responsible for modulating gastric cancer stem cell characteristics. We found that upregulated miR-106b

activated TGF-β/Smad signaling related to EMT and induced stem cell characteristics in CD44(+) gastric cancer stem-like cells.

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II. MATERIALS AND METHODS

1. Cell culture

MKN45 (KCLB 80103) and KATO III (KCLB 30103) gastric cancer cells were maintained in RPMI-1640 medium (Thermo Scientific, Rockford, IL, USA) supplemented with 10% FBS (Thermo Scientific) and 1% penicillin-streptomycin sulfate (Thermo Scientific). SW480 (KCLB-10228) colon cancer cells were maintained in DMEM medium (Thermo Scientific) supplemented with 10% FBS (Thermo Scientific) and 1% penicillin-streptomycin sulfate (Thermo Scientific). All cultures were maintained in a 37˚C incubator supplemented with 5% CO_2.

2. Flow cytometry analysis and fluorescence-activated cell sorting (FACS)

For flow cytometry, about 80% confluent cells in a 100-mm cell plate were washed with PBS and then cells were detached from plates using Trypsin-EDTA and centrifuged at 4˚C. Cell pellets were resuspended in HBSS (Gibco, Grand Island, NY, USA) supplemented with 1 mM HEPES (Gibco), 2% FBS and filtered with a 40-um mesh filter (BD Biosciences, San Jose, CA, USA). The cells were stained with a 400-fold dilution of anti-CD44-FITC (BD Biosciences) and incubated for 30 minutes in the 37˚C incubator supplemented with 5% CO_2. Then the cells were washed with HBSS and resuspended in HBSS supplemented with 1 mM HEPES, 2% FBS, and 1% penicillin-streptomycin sulfate. The cells were analyzed and sorted immediately with FACS Aria III (BD Biosciences).

3. Spheroid colony formation assay

FACS-sorted gastric cancer cells were planted in each well of ultra-low-attachment 24-well plates (Corning Life sciences, Corning, NY, USA) with DME/F12 medium (Thermo Scientific) supplemented with 20 ng/ml EGF

(R&D Systems, Minneapolis, MN, USA), 10 ng/ml basic FGF (R&D Systems), 1% ITS (Gibco), and 1% penicillin-streptomycin sulfate. Every three days, each well was examined using light microscopy.

4. RT-PCR and real-time PCR

Total RNA was extracted from culture cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using oligo(dT) primers and Superscript™ II reverse transcriptase (Invitrogen). PCR was carried out with a PCR Maxi Kit (iNtRON, Sungnam, Korea) according to the manufacturer's instructions. Amplification conditions included denaturation at 95˚C for 15 minutes, followed by 30 cycles of 30 seconds each at 95, 55, and 72˚C. PCR products were separated in 2% agarose gels. Real-time PCR was carried out with a PCR mixture containing 1 μmol/L of each primer and SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). The amplifications were conducted at 95˚C for 10 seconds and 60˚C for 60 seconds using a StepOnePlus™ real-time PCR system (Applied Biosystems). Each sample was examined in triplicate and the amount of PCR product was normalized with respect to *β-actin* as an internal control. PCR primers are shown in Table 1.

cDNA for miRNA assay was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) with miR-106b, miR-93, miR-25, and U6 specific primers (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR for miRNA quantification was carried out with TaqMan Universal Master Mix II (Applied Biosystems). The amplifications were conducted at 95˚C for 10 seconds and 60˚C for 60 seconds using an AB7500 real-time PCR system (Applied Biosystems). Each sample was examined in triplicate and the amount of PCR product was normalized with respect to U6 as an internal control.

Table 1. Primers sequence

Gene	Direction	Sequence (5' to 3')
Oct4	F	GACAACAATGAAAATCTTCAGGAGA
	R	TTCTGGCGCCGGTTACAGAACCA
Bmi	F	ATGTGTGTGCTTTGTGGAG
	R	AGTGGTCTGGTCTTGTGAAC
Nestin	F	AACAGCGACGGAGGTCTCTA
	R	TTCTCTTGTCCCGCAGACTT
ABCG2	F	CTGAGATCCTGAGCCTTTGG
	R	TGCCCATCACAACATCATCT
E-cadherin	F	TGCTTGGTTCACCAGTGGAT
	R	TTTTGTTGAGCAAGGCAACC
Vimentin	F	GAGAGGAAGCCGAAAACACC
	R	GCTTGGAAACATCCACATCG
Smad7	F	CGAGAGTGGGGAGGCTCTAC
	R	TTGTCCGAATTGAGCTGTCC
β -actin	F	TTGCCGACAGGATGCAGAAG
	R	AGGTGGACAGCGAGGCCAGG

5. Western blot analysis

Prepared cells were harvested after washing with PBS. Collected cells were lysed with buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor cocktail [Roche Molecular Biochemicals, Indianapolis, IN, USA]). The same amount of protein was boiled at 95˚C after adding SDS sample buffer (62.5 mM Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, β-

mercaptoethanol, and 0.002% bromophenol blue). Samples were loaded in 12% SDS-PAGE gels for Bmi, and Oct4; 8% SDS-PAGE gels for CD44, Nestin, ABCG2, E-cadherin, Vimentin, TGFβ-RI, TGF-βRII, Smad2, 3 and p-Smad2, 3 and then transferred to PVDF membranes.

Antibodies used include: CD44 (Abcam, Cambridge, MA, USA; 1:1000), Oct4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000), Bmi (Abcam; 1:1000), Nestin (Abcam; 1:1000), ABCG2 (Santa Cruz Biotechnology; 1:1000), E-cadherin (BD Bioscience; 1:1000), Vimentin (Millipore, Bedford, MA, USA; 1:1000), TGF-βRI (Abcam; 1:1000), TGF-βRII (Cell Signaling; 1:1000), Smad2 (Santa Cruz Biotechnology; 1:1000), Smad3 (Santa Cruz Biotechnology; 1:1000), p-Smad2/3 (Santa Cruz Biotechnology; 1:1000), and β-actin (Santa Cruz Biotechnology; 1:2000).

6. Immunofluorescence assay

Cells were seeded on glass coverslips in 6-well plates. After overnight incubation, the cells were washed with PBS three times, fixed in 10% formaldehyde for 10 minutes and permeabilized in 0.1% Triton 100X in PBS for 3 minutes. The cells were washed three times with PBS and blocked with 1% BSA in PBS buffer. The cells were incubated with the primary antibodies overnight at 4˚C. The slide was washed with PBS and incubated with FITC-conjugated secondary antibody for 1 hour at room temperature. The slides were mounted with DAPI. All samples were photographed using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

7. MicroRNA microarray

Total RNA of CD44(+) and CD44(-) MKN45 cells was prepared using TRIzol reagent according to the manufacturer's instructions. Microarray was performed using the Agilent Human miRNA 8 X 60K (Rel 16.0 V2)

platform (Agilent Technologies, Palo Alto, CA, USA). RNA hybridizations were performed with the Human microRNA Microarray Kit (Agilent Technologies) according to the manufacturer's protocol. Arrays were scanned on an Agilent C scanner. Images were quantified and data were processed using Agilent Feature Extraction Software (v 10.10.1.1). Raw data were extracted using the software provided by Agilent Feature Extraction Software (v 10.7.1.1). Selected miRNA gtotalGeneSignal value was transformed by logarithm and normalized by quantile method. The comparative analysis between test sample and control sample was carried out using fold-change. Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity.

8. MicroRNA inhibitor transfection

Cells were seeded into 6-well plates and incubated overnight. The cells were transfected with miR-106b inhibitor and negative control (Applied Biosystems) at 30nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 72 hours, the cells were harvested and RNA and protein were isolated.

9. Wound healing assay

Cells were seeded in 24-well plates, and incubated to attain 100% confluency. Linear scratches were made on the cell layers using pipette tips and washed with culture media. Cells were then incubated with serum-reduced medium for 72 hours. Gap closure was measured by light microscopy. Each sample was examined in triplicate.

10. Invasion assay

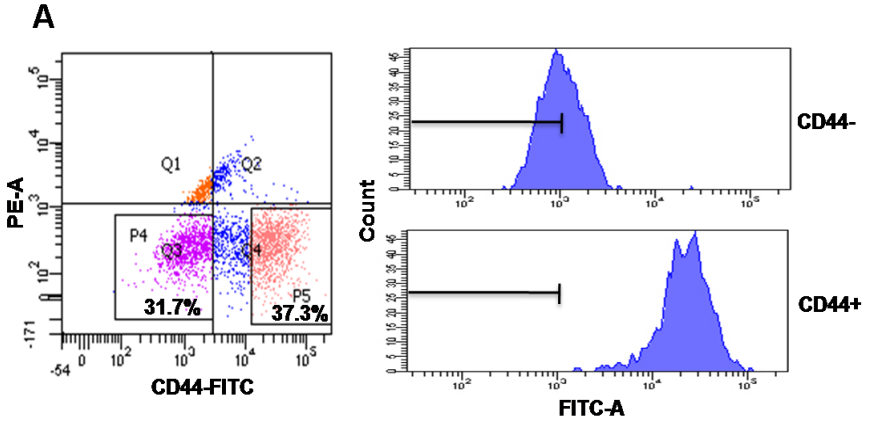
Twenty-four-well, 8.0-μm Transwells (BD Biosciences) were coated with diluted Matrigel (BD Biosciences) in PBS and dried for 6 hours. The cells

were resuspended in serum free media, and then $4x10^5$ cells were seeded in each top chamber. The lower chambers were filled with 10% FBS complete media for chemoattraction. To identify the effect of miR-106b knockdown, cells were transfected with miR-106b inhibitor and negative control. After 24 hours the cells were seeded into the inserts. Cell invasion chambers were incubated for 48 hours in a 37˚C incubator supplemented with 5% CO_2. Non-invaded cells on the upper surface were removed by cotton swab and the cells on the lower surface were fixed and stained with the Diff-Quik kit according to the manufacturer's instructions. Migrated cells on each insert were counted under a light microscope. Each sample was examined in triplicate.

III. RESULTS

1. CD44(+) cells show increased sphere colony formation than CD44(-) cells

Sphere colony formation assay is one approach to assess self-renewal potency of stem cells *in vitro*. FACS-sorted CD44(+) cells and CD44(-) cells (Fig. 1A) were grown in serum free conditions. After 1~2 weeks of incubations, CD44(+) cells were found to generate more and larger spheroid colonies than CD44(-) cells (Fig. 1B).



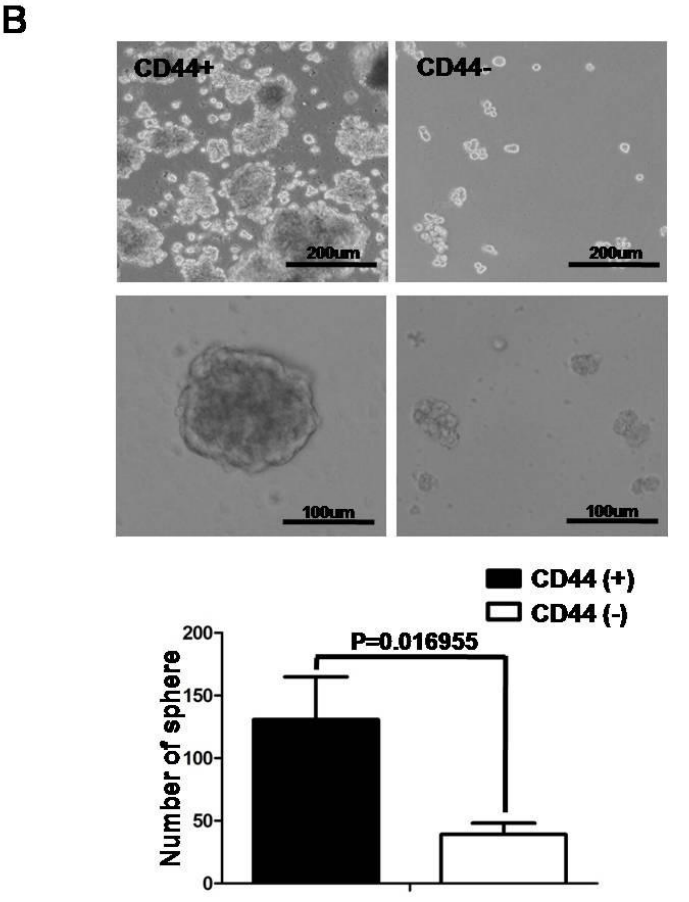
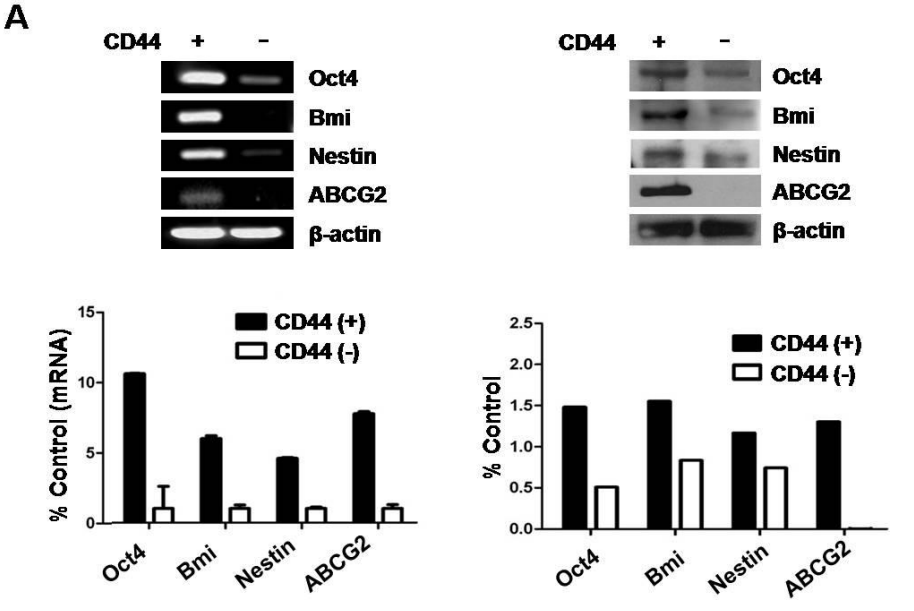


Figure 1. CD44(+) cells generate more and larger spheres than CD44(-) cells. (A) CD44 expression analysis in MKN45 cells by FACS. After fractionating cells into CD44(+) and CD44(-), degree of CD44 expression was analyzed by FACS. (B) Sphere formation abilities in CD44(+) cells and CD44(-) MKN45 cells were identified in low attachment plate with serum free media for 1~2 weeks. The graph showed the average number of spheres in triplicate.

2. CD44(+) cells show upregulated cancer stem cell marker expressions

RT-PCR and real-time PCR were used to evaluate the mRNA level of stem cell markers in CD44(+) and CD44(-) MKN45 cells. We used the

stem cell markers *Oct4, Bmi, Nestin,* and *ABCG2*. We found that the mRNA level of all stem cell markers was upregulated in the CD44(+) cells compared to the CD44(-) cells. Western blot analysis was performed to identify protein expression of the correspinding markers. The stem cell markers were overexpressed in CD44(+) cells with a similar pattern of mRNA expression (Fig. 2A). In addition, immunofluorescence assays were conducted to confirm CD44 and Oct4 expression in fractionated cells (Fig. 2B).



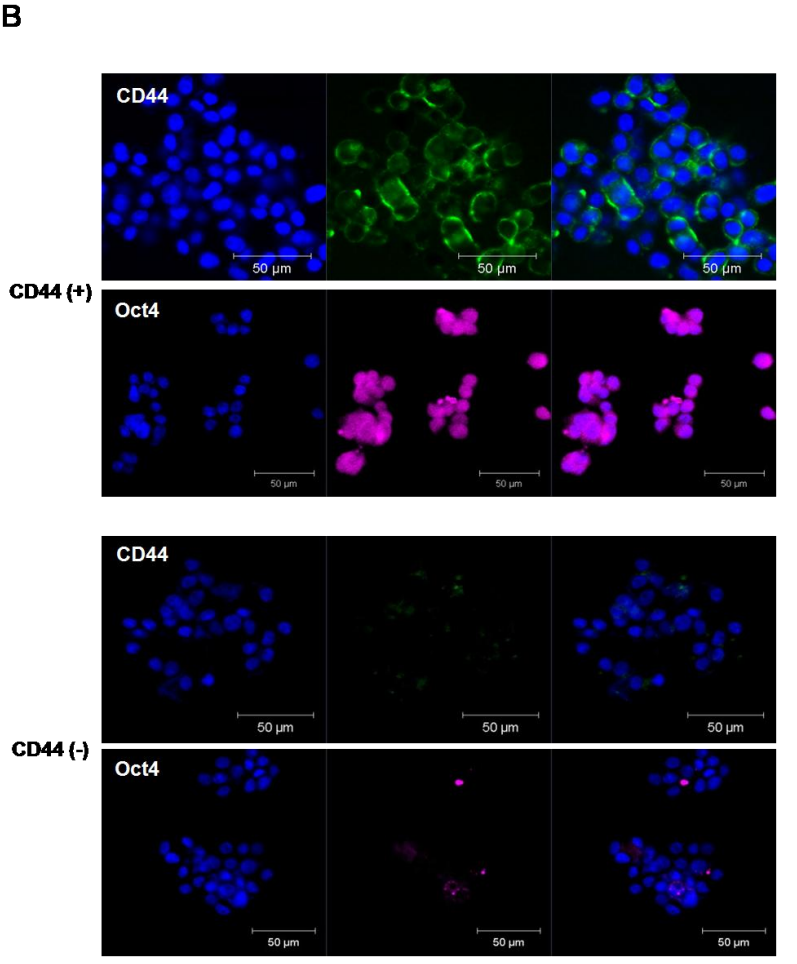


Figure 2. CD44(+) cells show stem cell marker overexpression

(A) mRNA expression of cancer stem cell markers; *Oct4, Bmi, Nestin, and ABCG2* in CD44(+) and CD44(-) MKN45 cells were measured by RT-PCR and real-time PCR (left panel). Protein expression of cancer stem cell markers in CD44(+) cells and CD44(-) cells were measured by western blot. The histogram displays the quantification of protein levels compared to β-actin control (right panel). (B) Expressions of CD44 and Oct4 were confirmed by immunofluorescence assay in CD44(+) and CD44(-) MKN45 cells with anti-CD44 (green, upper panel) and Oct4 (magenta, lower panel). The nucleus is stained with DAPI (blue).

3. Cell migration capacity is higher in CD44(+) cells

Cancer stem cells have higher invasion and migratory capacity which are thought to facilitate metastasis and growth. To validate the migration abilities of CD44(+) and CD44(-) cells, we conducted wound healing assay. Seventy two hours after making gaps, CD44(+) cells restored the wounds to greater degree than CD44(-) cells. The degree of migration of CD44(-) cells were show compared to the CD44(+) cells (Fig. 3).

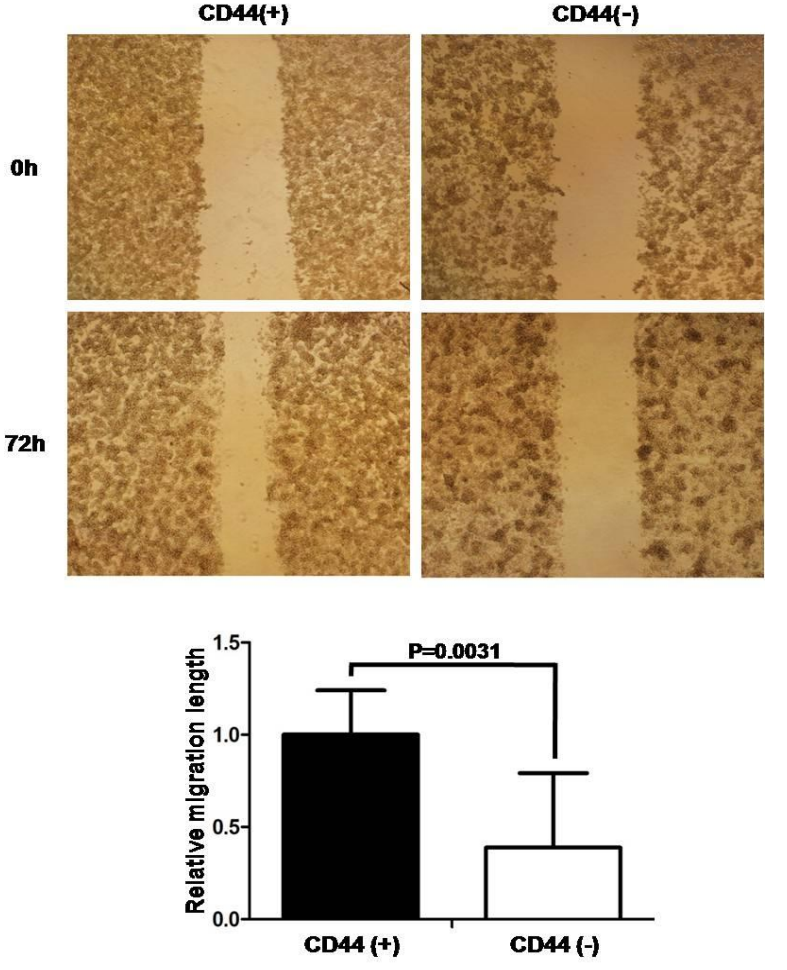
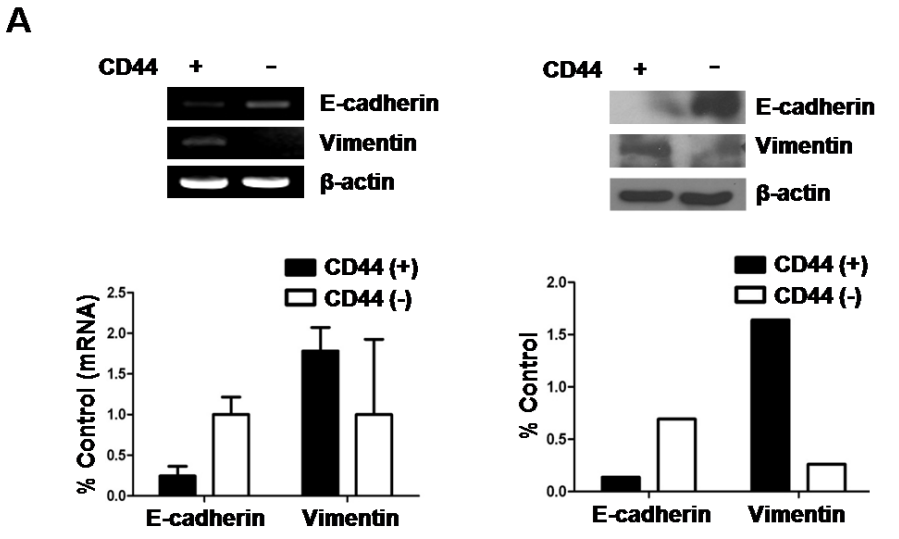


Figure 3. CD44(+) cells show higher rate of wounds closure magnitude compared to CD44(-) cells

Cell migration capacity was identified by wound healing assay. Degree of migrations were measured in CD44(+) and CD44(-) MKN45 cells at 72 hours time point. The graph showed averages of relative wound closure rates in triplicate.

4. CD44(+) cells shows mesenchymal characteristics

Cancer stem cells are thought to facilitate metastasis, so it is important to identify EMT characteristics relating to the mobility of cells. We used two well-known EMT markers; *E-cadherin*, an epithelial marker, and *vimentin*, a mesenchymal marker respectively. CD44(+) cells expressed higher levels of Vimentin than CD44(-) cells according to levels of both mRNA and protein. On the other hand, E-cadherin, a epitheilial cell marker was downregulated in the CD44(+) MKN45 cells (Fig. 4A, B).



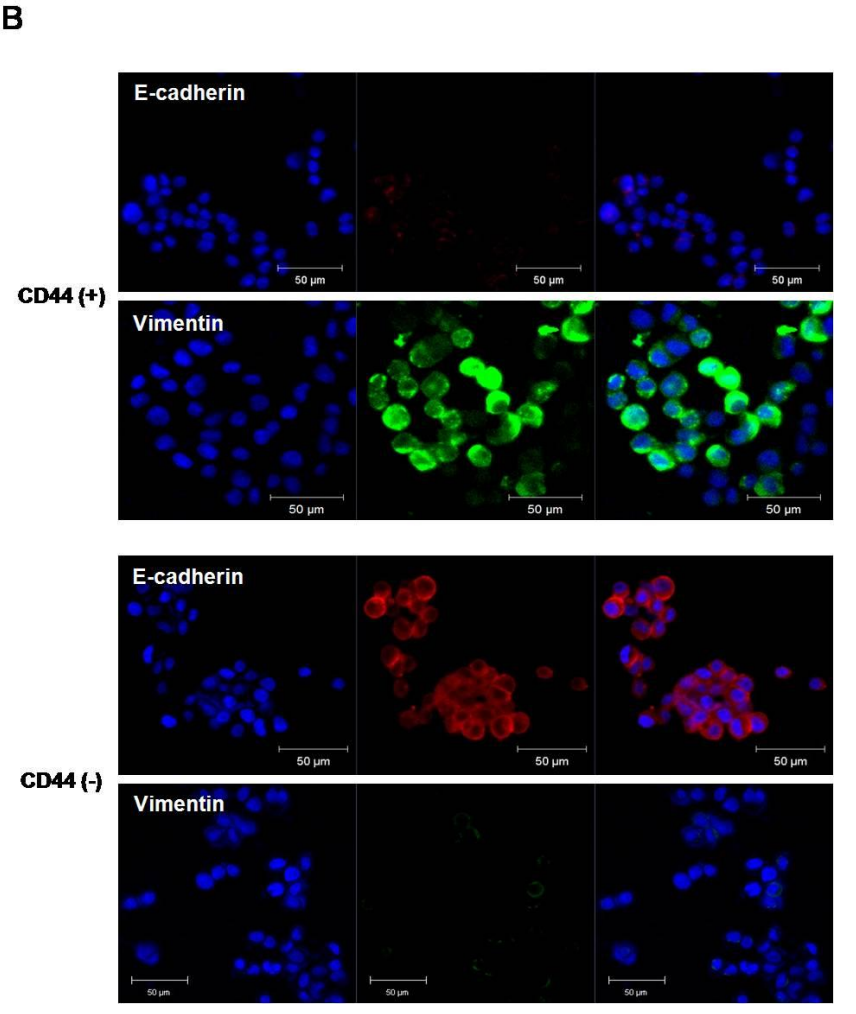


Figure 4. EMT markers are changed in CD44(+) MKN45 cells

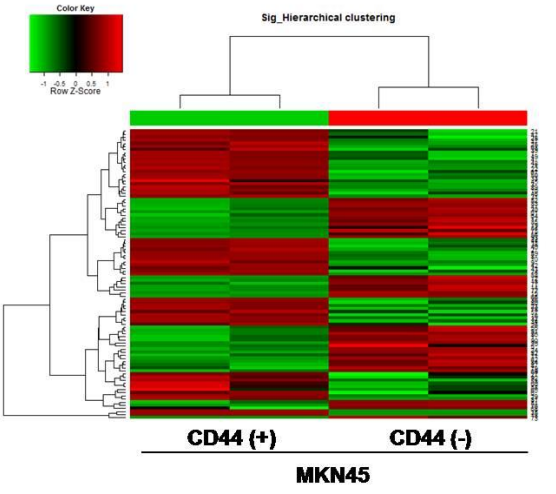
(A) mRNA expression of EMT markers; *E-cadherin* and *vimentin* in CD44(+) and CD44(-) MKN45 cells were measured by RT-PCR and real-time PCR that was normalized with respect to β-actin (left panel). Protein expression of EMT markers in CD44(+) cells and CD44(-) cells were measured by western blot. The histogram displays the quantification of protein levels compared to β-actin control (right panel). (B) Expressions of E-cadherin and vimentin were confirmed by immunofluorescence assay in CD44(+) and CD44(-) MKN45 cells cells with anti-E-cadherin (red, upper

panel) and Vimentin (green, lower panel). The nucleus is stained with DAPI (blue).

5. CD44(+) cells show upregulation of miR-106b family in microarray

miRNA microarray analysis was performed on RNA isolated from CD44(+) cells and CD44(-) MKN45 cells in duplicate. Figure 5A shows miRNAs that were upregulated in CD44(+) cells as compared to CD44(-) cells in a statistically significant manner between the two groups. We found that miR-106b, miR-93, and miR-25—the components of the miR-106b family—were upregulated in CD44(+) cells compared to CD44(-) (Fig. 5A). To validate the microarray data, we measured miR-106b family levels by real-time PCR on an independent set of RNA isolated from MKN45, KATO III, and SW480 CD44(+) and CD44(-) cells respectively (Fig. 5B). We confirmed that the miR-106b family is overexpressed in CD44(+) cells and the expression level gap between CD44(+) and CD44(-) cells is widest among these three miRNAs. This prompted us to carry on further study on the regulatory mechanism of miR-106b in gastric cancer stem-like cells.

A



Gene name	Fold change	p-value
hsa-miR-634	3.766183	0.048217
hsa-miR-193a-3p	3.094264	0.005929
hsa-miR-18b-3p	2.546766	0.0186
hsa-miR-425-5p	2.043163	0.001469
hsa-miR-221-3p	2.026288	0.018695
hsa-miR-424-5p	1.996587	0.005721
hsa-miR-93-5p	**1.794756**	**0.003362**
hsa-miR-15a-5p	1.793585	0.049413
hsa-miR-25-3p	**1.779954**	**0.043274**
hsa-miR-30e-5p	1.735466	0.043003
hsa-miR-210	1.692317	0.042062
hsa-miR-30c-5p	1.68477	0.012501
hsa-miR-149-5p	1.616103	0.025772
hsa-miR-106b-5p	**1.614756**	**0.03505893**

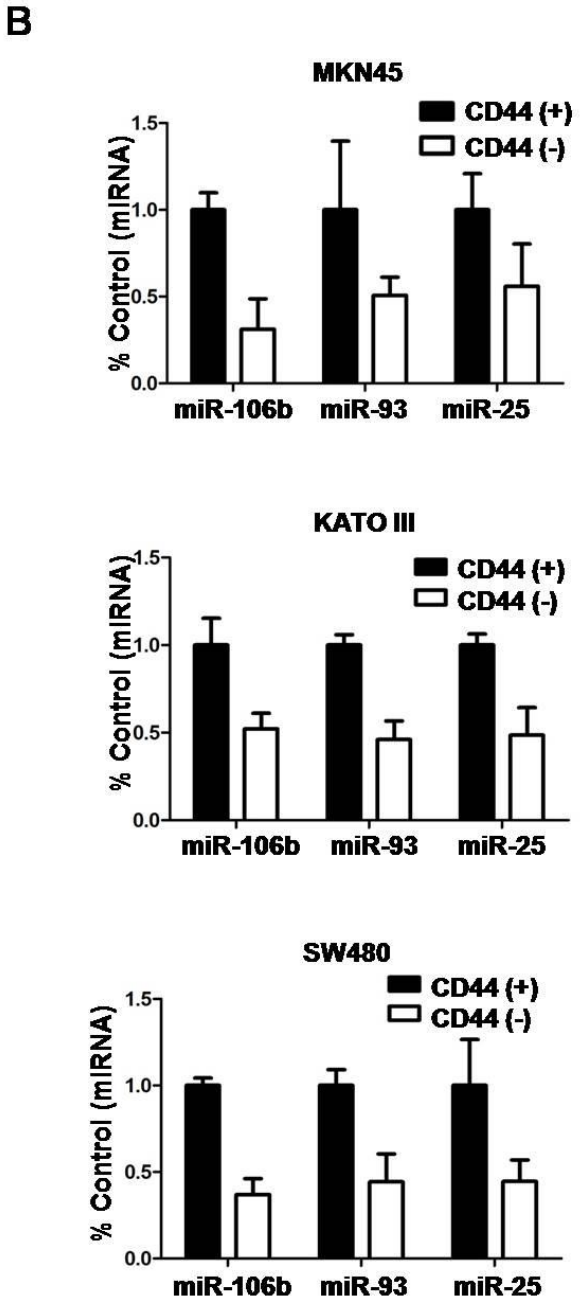


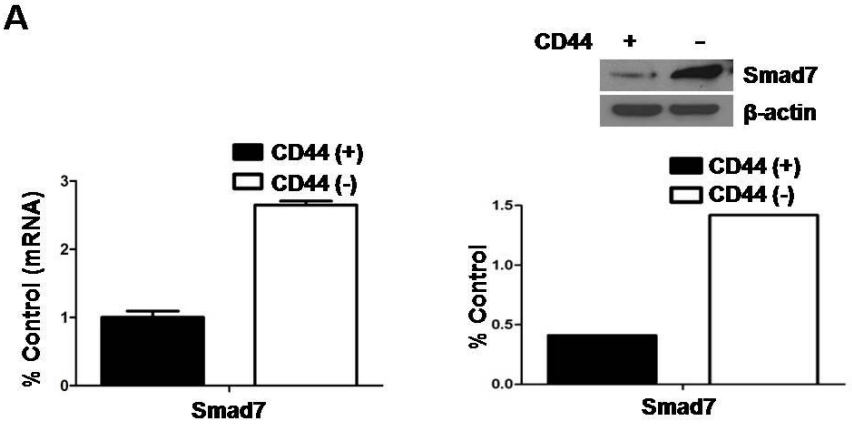
Figure 5. miR-106b family are upregulated in CD44(+) cells

(A) Hierarchical clustering shows miRNA profiles in CD44(+) and CD44(-)

MKN45 cells. The colors indicate miRNA expression as exhibit in the color key. miRNAs upregulated in CD44(+) cells listed in the table (fold change>1.5, $p<0.05$). (B) Expression of miR-106b family was identified using real-time PCR in three cell lines. Cells were sorted according to CD44 expression by FACS.

6. miR-106b regulates TGF-β/Smad signaling through the inhibitory protein Smad7

miR-106b is known to activate the TGF-β/Smad signaling pathway by targeting Smad7. Smad7 inhibits phosphorylation of Smad2/3 and suppresses expression of the TGF-β receptor as an inhibitor protein of Smad signaling. To examine the expression level of Smad7 and TGF-β/Smad signal molecules, we performed real-time PCR and western blot in CD44(+) and CD44(-) MKN45 cells. Smad7 was significantly decreased in CD44(+) cells at mRNA and protein level (Fig. 6A). TGF-β receptors and p-Smad2/3, the active form of Smad2/3, was suppressed in CD44(-) cells. The downstream transcription factor, Slug was also upregulated in CD44(+) cells compare to CD44(-) cells (Fig. 6B).



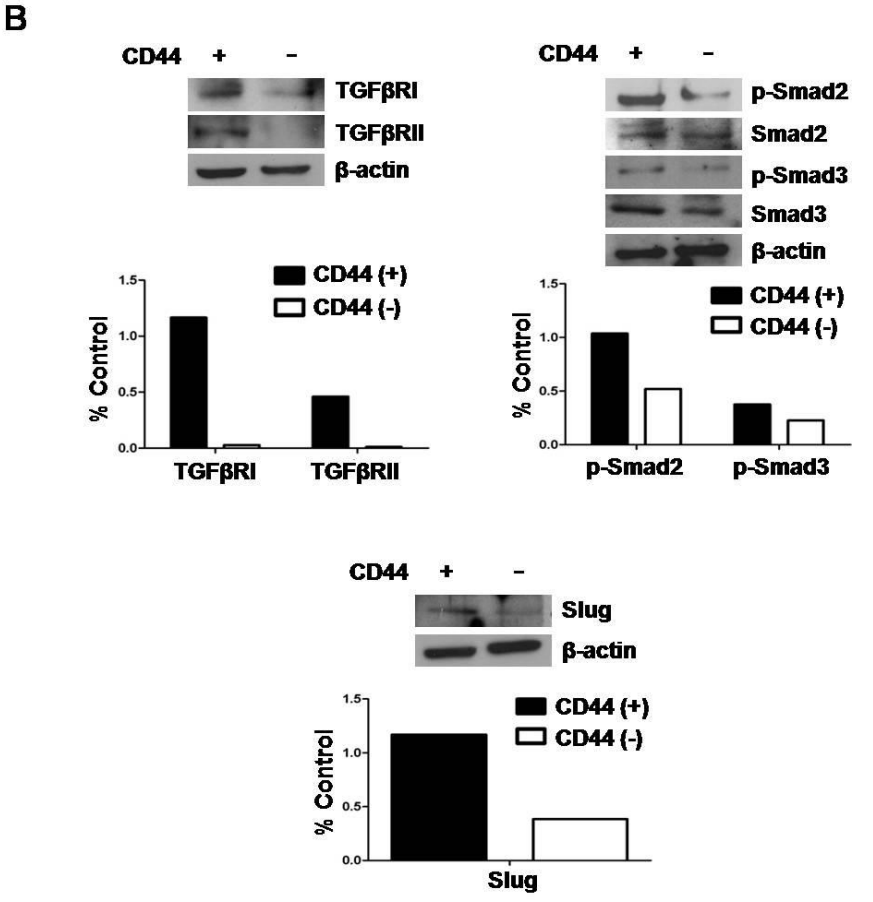
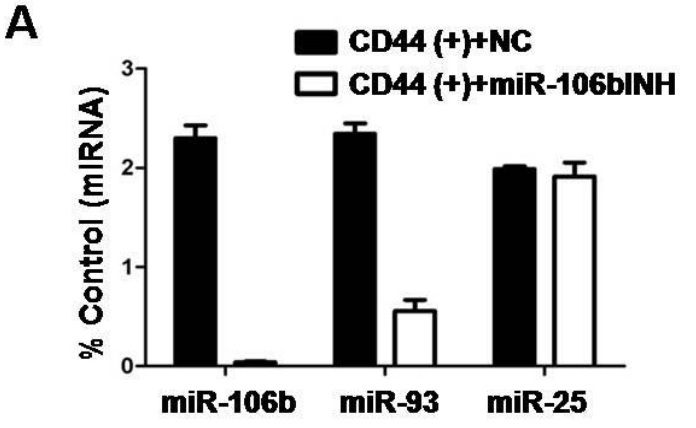


Figure 6. Downregulated Smad7 activates TGF-β/Smad signaling molecules (A) mRNA expression of Smad7 in CD44(+) and CD44(-) MKN45 cells were measured by real-time PCR (left panel). Protein expression of Smad7 in CD44(+) and CD44(-) MKN45 cells were measured by western blot. The histogram displays the quantification of protein levels compared to β-actin control (right panel). (B) Protein expression of TGF-β/Smad signaling molecules in CD44(+) cells and CD44(-) cells were measured by western blot. The histogram displays the quantification of protein levels compared to β-actin control except phosphorylated Smad2 and 3. Expression of p-Smad2 and 3 were compared against Smad 2 and 3 expression. (TGFβRI; TGF-β receptor I, TGFβRII; TGF- β receptor II)

7. Expression of TGF-β/Smad signaling molecules repressed by miR-106b inhibition

To study the function of miR-106b, we transfected miR-106b inhibitor and a negative control in CD44(+) MKN45 cells. We conducted real-time PCR to confirm miRNA knockdown. miR-106b and miR-93 levels were decreased, but miR-25 remained unchanged (Fig. 7A). Expression change of Smad7 by miR106b downregulation was identified by real-time PCR and western blot. Inhibition of miR-106b caused Smad7 overexpression in CD44(+) cells (Fig. 7B). The CD44(+) cells suppressed of miR-106b expression by its inhibitor also showed decreased expression of TGF-β receptors, phosphorylated Smad2/3, and Slug (Fig. 7C).



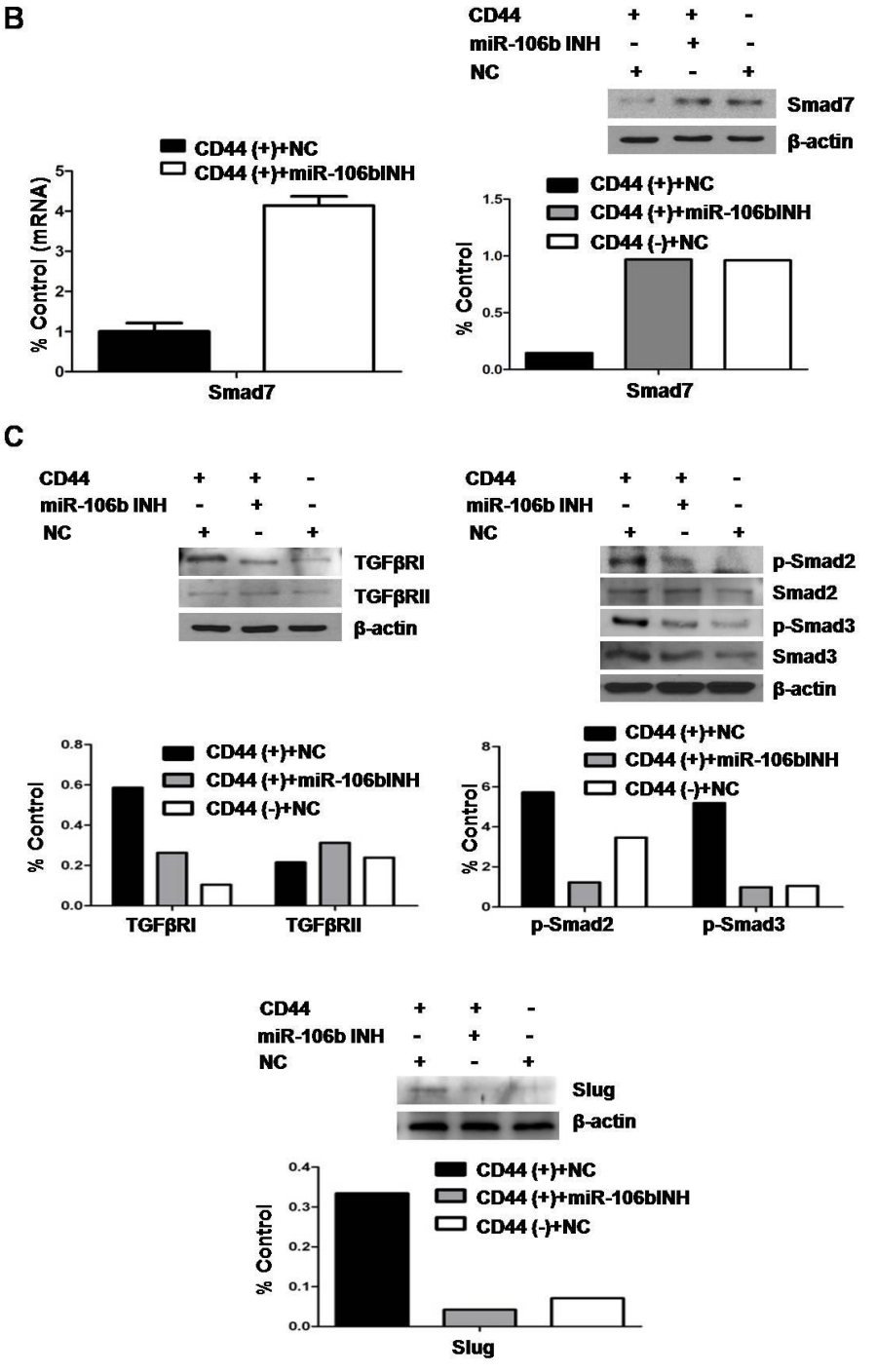


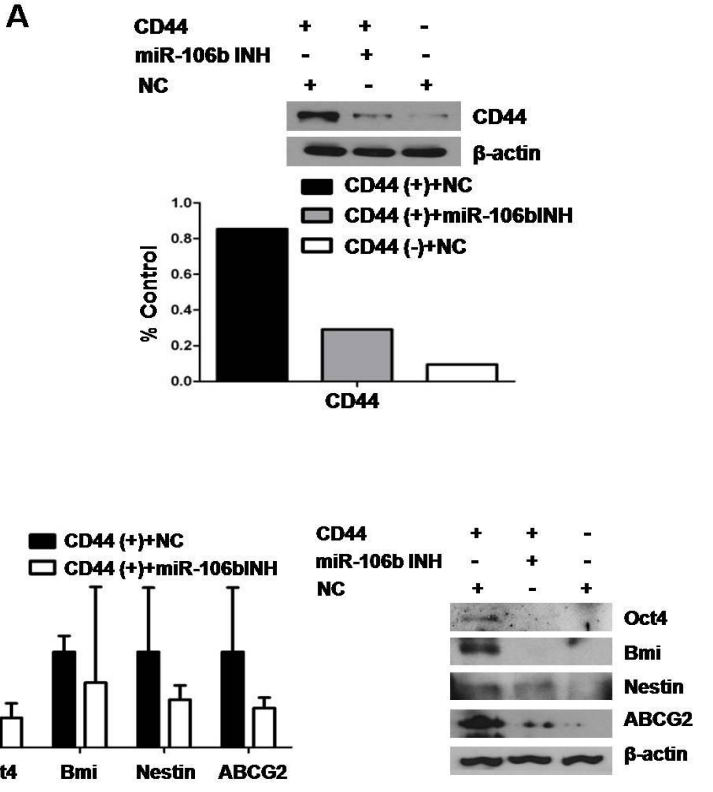
Figure 7. Knockdown of miR-106b suppresses TGF-β/Smad signaling

(A) miRNA 106b family expression was identified by real-time PCR in CD44(+) MKN45 cells transfected with miR-106b inhibitor and negative control. (B) mRNA expression of Smad7 in CD44(+) MKN45 cells transfected with miR-106b inhibitor and negative control were measured by real-time PCR (left panel). Protein expression of Smad7 in CD44(+), CD44(-) MKN45 cells transfected with negative control and CD44(+) cells transfected with miR-106b inhibitor. were measured by western blot. The histogram displays the quantification of protein levels compared to β-actin control (right panel). (C) Expression of TGF-β/Smad signaling molecules were validated by western blot in CD44(+), CD44(-) MKN45 cells transfected with negative control and CD44(+) cells transfected with miR-106b inhibitor.. The histogram displays the quantification of protein levels compared to β-actin control. (NC; negative control, miR-106bINH; miR-106b inhibitor, TGFβRI; TGF-β receptor I, TGFβRII; TGF- β receptor II)

8. Expression of cancer stem cell markers and sphere colony forming ability were suppressed by miR-106b inhibition in CD44(+) cells

Next, we investigated the regulation of cancer stem cell characteristics by miR-106b. The expression of cancer stem cell markers was measured by real-time PCR and western blot. First, expression of CD44 protein was suppressed in CD44(+) cells transfected with miR-106b inhibitor (Fig. 8A). And cancer stem cell markers were relatively downregulated in the CD44(+) cells transfected with miR-106b inhibitor compared to CD44(+) control cells (Fig. 8B). Sphere colony assay was conducted to validate the self-renewal capacity in CD44(+) and CD44(-) cells and CD44(+) transfected with miR-106b inhibitor. miR-106b knockdown decreased sphere forming ability in CD44(+) cells (Fig. 8C). The expression patterns of those markers in the CD44(+) cells transfected with miR-106b inhibitor were in similar pattern to CD44(-) cells. And miR-106 downregulated

CD44(+) cells formed fewer spheres than CD44(+) control. These results suggest that miR-106b affects expression of stem cell genes and self-renewal capacity in gastric cancer stem cells.



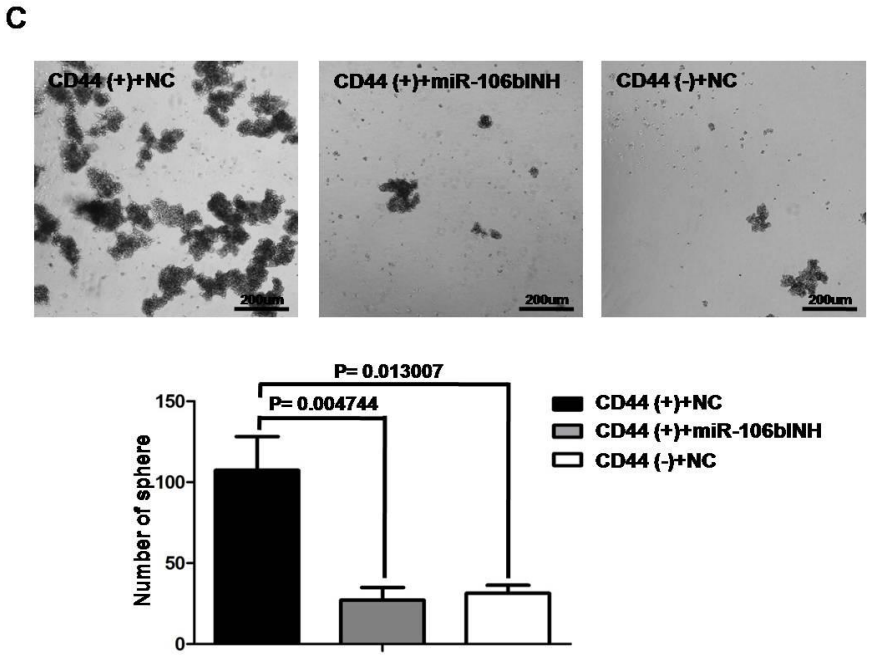


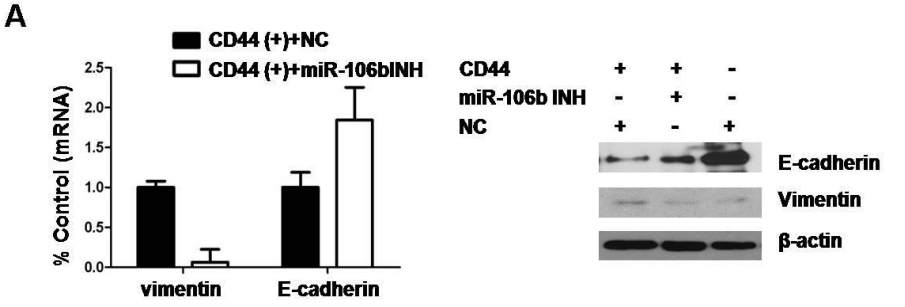
Figure 8. Knockdown of miR-106b suppresses expression of cancer stemness gene and sphere forming ability

(A) CD44 expression was measured by western blot in CD44(+), CD44(-) MKN45 cells transfected with negative control and CD44(+) cells transfected with miR106b inhibitor. (B) Expression of cancer stem cell markers; *Oct4*, *Bmi*, *Nestin*, and *ABCG2* were identified using real-time PCR and western blot in CD44(+), CD44(-) MKN45 cells transfected with negative control and CD44(+) cells transfected with miR106b inhibitor. (C) Sphere formation abilities in CD44(+), CD44(-) MKN45 cells transfected with negative control and CD44(+) cells transfected with miR-106b inhibitor were identified in low attachment plate with serum free media for 1 weeks. The graph showed the average number of sphere in triplicate. (NC; negative control, miR-106bINH; miR-106b inhibitor)

9. EMT markers showed changed expression and high invasiveness is inhibited by miR-106b knockdown in CD44(+) cells

To identify EMT markers expression, we perform real-time PCR and western blot. E-cadherin was upregulated but vimentin was downregulated in miR-106b downregulated CD44(+) cells (Fig. 9A).

We performed an invasion assay to identify phenotypic changes in cells with downregulated TGF-β/Smad signaling molecules resulting from miR-106b inhibitor transfection. The number of invading cells among each group was counted and compared against the number of negative control transfected CD44(+) cells. The number of CD44(+) cells able to pass through the membrane was approximately double that of CD44(-) cells. Also, miR-106b inhibitor transfected CD44(+) cells demonstrated decreased invasion ability (Fig. 9B). miR-106b interference effectively suppresses mesenchymal gene expression and the high invasiveness of CD44(+) cells.



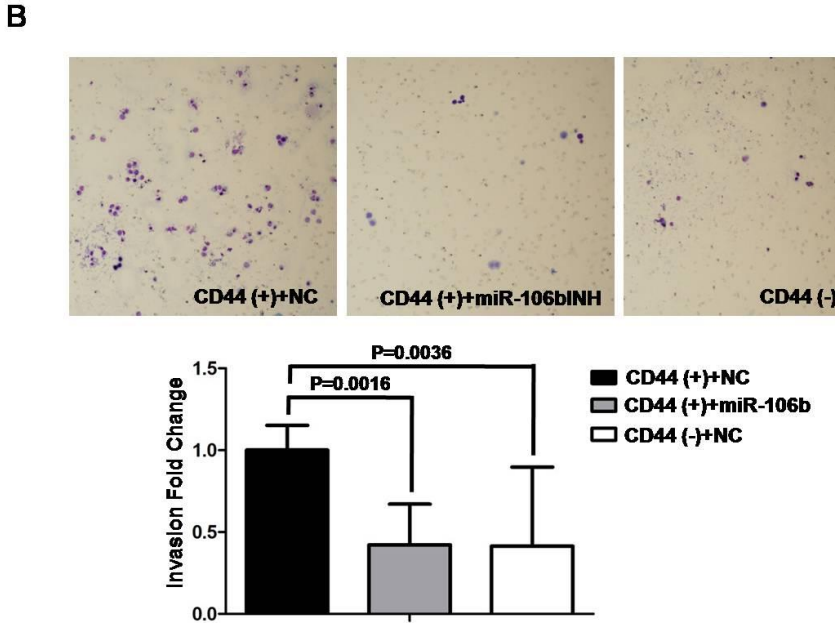


Figure 9. Knockdown of miR-106b suppresses mesencymal marker and cell invasiveness

(A) Real-time PCR and western blot were conducted to investigate expression of EMT markers; *E-cadherin* and *vimentin* in CD44(+), CD44(-) MKN45 cells transfected with negative control and CD44(+) cells transfected with miR-106b inhibitor. (B) Cell invasion ability was compared by Matrigel coated invasion assay in CD44(+), CD44(-) MKN45 cells transfected with negative control and CD44(+) cells transfected with miR-106b inhibitor. Invaded cells were counted and converted to the graph. (NC; negative control, miR-106bINH; miR-106b inhibitor)

IV. DISCUSSION

Many studies have established the existence of cancer stem cells in various tumors. Cancer stem cells can generate new tumors and can be a cause for drug resistant and tumor metastasis so elimination of cancer stem cells has attracted attention as a new approach of cancer therapy [21].

In this study, we used CD44 as a surrogate marker of gastric cancer stem cells. We examined cancer stem cell surface markers, CD44 and EpCAM, that were used widely, but only cells sorted by CD44 expression showed unique cancer stem cell phenotypes in gastric cancer cells. FACS-sorted CD44(+) cells showed many characteristics of cancer stem cells as compared with CD44(-) cells. The self-renewal capacity of CD44(+) cells was examined by sphere formation ability, and the expression level of genes upregulated in many cancer stem cells was validated at the mRNA and protein level. Also, EMT properties were investigated in terms of molecular expression and phenotype between CD44(+) and CD44(-) cells. As a result, CD44(+) cells showed a shift towards mesenchymal cell characteristics as expected of cancer stem cells.

Next, we analyzed the miRNA expression profiles in gastric cancer cells as a new approach to determine the regulatory mechanisms relating to cancer stem cell development. CD44(+) and CD44(-) cells showed a different pattern of miRNA expression. We have chose the miR-106b family composed of miR-106b, miR-93, and miR-25 among the many selected candidates based on miRNA microarray data because all three miRNAs were consistently upregulated in CD44(+) cells significantly. miR-106b family is one of upregulated miRNAs in high grade cancer [22,23]. miR-106b family is located in intron 13 in *Mcm7* gene at chromosome 7q22. miR-106b family have two paralogs, miR-17-92 and miR-106a-363 that regulate cancer related genes and miR-106b family is less studied in these paralogs. miR-

106b family has been studied mainly in cell cycle regulation. As p21, cyclin-dependent kinase inhibitor is one of target genes of miR-106b family. miR-106b family inhibits p21 expression by binding its 3'-UTR and it causes cancer cells proliferation in several tumors.

TGF-β signaling pathway has been identified that it plays a role in many cellular pathways. Also TGF-β signaling implicated in tumor progression and cancer stem cell characteristics [24]. In breast cancer, TGF-β treatment cause the transition $CD24^+$cells to $CD24^-$ cells showed cancer stem cell characteristics [25]. Activation of Smad2/3, TGF- β signaling pathway transducers, maintains the pluriptency in human embryonic stem cells [26].

Recently, miR-106b was also known to regulate EMT by the TGF-β/Smad signaling pathway implicated in cancer stem-like cell development [24,27,28]. The newly found target gene of miR-106b is Smad7 which acts as an inhibitor of TGF-β/Smad signaling. Smad7 binds to TGF-β receptor I and interrupts recruitment of TGF-β receptor I and II. Smad7 degrades TGF-β receptor I by interacting with ubiquitin ligase [29]. Smad7 additionally blocks Smad2/3 activation by interfering with their phosphorylation. As a result, the downstream transcription factor of TGF-β/Smad signaling, Slug, is downregulated [30].

In this study, CD44(+) gastric cancer stem-like cells, with upregulated miR-106b, showed activated TGF-β/Smad signaling and increased expression of Slug. And we confirmed that knockdown of miR-106b caused inhibition of TGF-β/Smad signaling pathway leading to inhibition of EMT and stem cell characteristics. Cancer stemness genes were downregulated and self-renew capacity was inhibited in CD44(+) cells by miR-106b knockdown. Also, miR-106b inhibition repressed upregulated mesenchymal characteristics of CD44(+) cells.

Taken together, our results indicate that miR-106b upregulated in CD44(+) gastric cancer stem-like cells, makes to retain cancer stem cell characteristics

by modulation of the TGF-β/Smad signaling pathway. miR-106b can be a new marker of gastric cancer stem cells and may be a promising target for cancer treatment.

V. CONCLUSION

Recently, miRNA is becoming an important regulator of cellular processes and also carcinogenesis. And cancer stem cells have many unknown mechanisms. We tried to find new effective marker of cancer stem cells in gastric cancer. We found that miR-106b regulates cancer stem cell characteristics through TGF-β/Smad signaling pathway in gastric cancer stem cells.

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<ABSTRACT(IN KOREAN)>

CD44양성 위암세포에서 TGF-β/Smad 신호전달기전을 통한 miR-106b의 암줄기세포 특성 조절

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유 다 연

암줄기세포는 새로운 종양을 형성할 수 있는 능력을 가진 세포로 암의 전이와 재발에 연관이 있다고 알려져있다. 그러나 암줄기세포의 발달에 연관된 수많은 기전에 관하여는 여전히 완벽히 밝혀내지 못하였다. 최근의 연구 결과들에서 마이크로 RNA는 암줄기세포의 특성을 갖게하고 암줄기세포를 형성하는데 중요한 조절자로 여겨진다.

이 연구의 목적은 CD44 양성 위암줄기양세포의 특징을 조절하는 miRNA를 찾아내는 것이다. 우리는 암줄기세포 표지자인 CD44의 발현에 따라 FACS를 이용하여 위암세포주에서 위암줄기양 세포를 분리하였다. CD44(+) 세포는 CD44(-) 세포보다 더 크고 많은 sphere를 형성하였으며, 암줄기세포에서 높게 발현하는것으로 알려진 표지자들이 mRNA와 단백질 수준에서 모두 CD44(-) 세포보다 높은 발현을 보였다. 또한 중간엽세포 표지자인 vimentin은 CD44(-)세포에 비해 CD44(+) 세포에서 높은 발현을 보였고, 반대로 상피엽세포 표지자인 E-cadherin은 그 발현이 낮았다. 또한 CD44(+) 세포가

CD44(-) 세포보다 높은 이동 능력을 보였다. 마이크로 RNA array 결과 miR-106b family가 CD44(+) 세포에서 높은 발현을 보였다. TGF-β/Smad 신호전달기전에서 억제자로 작용하는 miR-106b의 표적 물질인 Smad7은 CD44(+) 세포에서 발현이 감소되어 있었다. 또한 TGF-β/Smad 신호전달을 구성하는 유전자들 역시 miR-106b의 작용에 의해 CD44(+) 세포에서 활성화되어 있었다. miR-106b를 억제하면 TGF-β/Smad 신호가 억제되었고, 세포의 침투력 역시 감소하였다. 이상의 연구는 CD44(+) 위암 세포가 암줄기세포의 특성과 상피중간엽이행 특성을 보이며, miR-106b가 TGF-β/Smad 신호전달기전을 통하여 이러한 특징들을 조절하는 조절자로서 작용한다는 것을 보여준다. 이번 연구 결과로 miR-106b를 타겟으로 하는것은 위암에서의 새로운 암 치료법의 하나로 고려될 수 있다고 생각한다.

핵심되는 말 : 위암, 줄기세포, 상피중간엽이행, 마이크로 RNA

PUBLICATION LIST