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Research article

MiRNA-20b/SUFU/Wnt axis accelerates gastric cancer cell proliferation, migration and EMT

Yin Peng [a,b,1,**], Ying Qin [d,1], Xiaojing Zhang [a,b,c], Shiqi Deng [a], Yuan Yuan [a], Xianling Feng [a], Wangchun Chen [a], Fan Hu [a], Yuli Gao [a], Jieqiong He [a], Yulan Cheng [e], Yanjie Wei [f], Xinmin Fan [a], Hassan Ashktorab [g], Duane Smoot [h], Song Li [i], Stephen J. Meltzer [e], Shutong Zhuang [d], Na Tang [j], Zhe Jin [a,b,*]

[a] Guangdong Key Laboratory for Genome Stability & Disease Prevention, Department of Pathology, Shenzhen University School of Medicine, Shenzhen, Guangdong 518060, China
[b] Guangdong Provincial Key Laboratory of Regional Immunity and Diseases, Department of Pathology, Health Science Center, Shenzhen University, Shenzhen, Guangdong, 518055, China
[c] Department of Pathology, Guangdong Province Key Laboratory of Molecular Oncologic Pathology, China
[d] Department of Gastrointestinal Surgery, Shenzhen Second People's Hospital, Shenzhen, Guangdong 518000, China
[e] Department of Medicine/GI Division, Johns Hopkins University School of Medicine and Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD 21287, USA
[f] Center for High Performance Computing, Shenzhen Institutes of Advanced Technology, Shenzhen, Guangdong, 518000, China
[g] Department of Medicine and Cancer Center, Howard University, College of Medicine, Washington, DC 20060, USA
[h] Department of Medicine, Meharry Medical Center, Nashville, TN 37208, USA
[i] Shenzhen Science & Technology Development Exchange Center, Shenzhen Science and Technology Building, Shenzhen, Guangdong, 518055, China
[j] Department of Pathology, Shenzhen People's Hospital (The Second Clinical Medical College, Jinan University, The First Affiliated Hospital, Southern University of Science and Technology), Shenzhen 518020, Guangdong, China

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ABSTRACT

Previous research has found that miRNA-20b is highly expressed in gastric cancer (GC), however, its function and underlying mechanism are not clear. Wnt signaling pathway, implicated in tumorigeneisis, is activated in more than 30% of GC. We would like to characterize the biological behavior of miRNA-20b in terms of modulating Wnt/β-catenin signaling and EMT. We showed that miRNA-20b inhibitors suppressed Topflash/Fopflash dependent luciferase activity and the β-catenin nuclear translocation, resulting in inhibition of Wnt pathway activity and EMT. SUFU, negatively regulating Wnt and Hedgehog signaling pathway, was proved to be targeted by miRNA-20b. Moreover, additional knockdown of SUFU alleviated the inhibitory effect on Wnt pathway activity, EMT, cell proliferation/migration and colony formation caused by miRNA-20b inhibition.

In summary, miRNA-20b is an oncogenic miRNA and promoted cell proliferation, migration and EMT in GC partially by activating Wnt pathway via targeting SUFU.

1. Introduction

GC, causing second cancer-related mortality, is a very common cancer worldwide, [1]. Over 1,000,000 new GC cases and 783,000 deaths were estimated in 2018 globally, with 5.7% incidence rate and 8.2% mortality rate [2]. Great effort has been put to improve the overall survival of GC patients, however the prognosis of late GC patient is still dismal. Thereby, it is crucial to elucidate the oncogenic mechanism of GC, which will help to search for biomarkers for earlier detection and new therapeutic targets at molecular level.

Though miRNAs (microRNAs) were discovered in the last century, they become promising targets for diagnosis, prognosis and therapeutics for GC in the last decade. Previous research has found that in GC miRNA-20b is overexpressed and its high level predicts poor overall survival in GC patients [3, 4, 5, 6]. However, the action role of miRNA-20b is largely unknown. In this essay, we would like to determine the tumorigenesis

* Corresponding author.
** Corresponding author.
E-mail addresses: ypeng@szu.edu.cn (Y. Peng), zhejin@szu.edu.cn (Z. Jin).
[1] These authors contributed equally to this paper.

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mechanism of miRNA-20b and its target gene mediating the oncogenic effect.

The canonical Wnt signaling pathway plays an essential role in regulating cell proliferation, development, and differentiation [7]. Dysregulated canonical Wnt pathway has been implicated in GC [8]. Wnt signaling activation results from the degradation of APC (adenomatous polyposis coli) destruction complex and subsequent nuclear translocation of β-catenin. β-catenin nuclear accumulation is observed in 30–50% GC [9]. It is reported that *H. pylori* infection dysregulates Wnt signaling pathway by interacting with E-cadherin [10] and activating PI3K/Akt pathway [11]. In addition, TCGA study highlights the significance of Wnt pathway mutations in GCs [12]. However, the mechanism underlying dysregulated Wnt/β-catenin signaling activation is not clear.

In this study, we would like to elucidate the biological function of miRNA-20b and its involvement in Wnt/β-catenin signaling activation in GC. We hypothesize that miRNA-20b enhances Wnt pathway activity and induces EMT (Epithelial-Mesenchymal Transition). Furthermore, we would like to approve that SUFU (Suppressor of Fused) is the target of miRNA-20b. Moreover, SUFU might mediate Wnt activation, EMT and the tumorigenesis role of miRNA-20b. Together, we speculate that miRNA-20b promotes cell proliferation, migration and EMT by inducing Wnt signaling activation via targeting SUFU.

2. Material and methods

2.1. Cell culture

HFE145, immortalized normal human gastric epithelial cells, were obtained from Dr Smoot at Howard University and gastric cancer cells (BGC823) were from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), respectively. Cell identity has been confirmed by STR testing. These cells were cultured in 90%DMEM (HyClone,#SH30243.01) plus 10% fetal bovine serum (Gibco,#10270) and Plasmocin (mpp-39-03,invivogen).

2.2. RNA extraction and PCR for miRNA

mirVana RNA isolation kit (Ambion, #AM1561) was used to extract total RNAs from tissues and cultured cells according to the instruction. RNAs were stored at -80 °C until use. Specific primers (#PN4427975 60C04, #PN4427975 82D07) for miRNA-20b detection were synthesized by Applied Biosystems. TaqMan MicroRNA Assays were used to quantify miRNA expression (Applied Biosystems, #4428175). RNU6B was used as an internal control.

2.3. MiRNA mimics, inhibitors and siRNAs transfection

Three isolate siRNAs for knockdown of SUFU were synthesized by Ribobio (Guangzhou, China). 60nM of mixed three siRNAs was transfected in GC cells using RNAiMAX (Invitrogen, #13778150) 48 hrs before RNA and protein extraction. SUFU siRNAs were listed:5′-CGG CCT GAG TGA TCT CTA T-3′, 5′-GATCCA CAC CTG CAA GAG A-3′ and 5′-GCA GCT TGA GAGCGT ACA T-3'. NC (Negative control) siRNA served as a negative control.

MiRNA mimics and miRNA inhibitors were obtained from Dharmacon (Lafayette, CO, USA). Cells with 30–50% confluency were transfected with either miRNA mimics or miRNA inhibitors (60nM) or cotransfected with inhibitors and 60nM siRNAs using Lipofectamine RNAiMAX (Invitrogen,#13778150). Forty-eight hrs after transfection, RNA or protein was extracted. Nonspecific controls (NSC) for inhibitors, mimics served as negative controls.

2.4. Cell proliferation

CCK8 (Cell Counting Kit-8) assay (Dojindo, #GC709) was used for measurement of cell proliferation according to the manufacturer's manual. Cells were plated at a density of 1000 cells/well in the format of 96-well plate. After 1, 3 and 5 days of growth in the incubator, 10 μl of CCK-8 reagent was added into each well and then incubated for 1 hr. A microplate photometer (Molecular Devices, Sunnyvale, USA) was used to detect the Optical Density at 450nm.

2.5. Cell migration assay

For migration assay, 48 hrs post transfection, cells were harvested and suspended in serum-free DMEM medium. 5×10^4 cells were seeded into transwell chamber with an 8.0 mm pore membrane (Corning, #3422). The chambers were put into wells and then incubated in medium with 20% FBS for 24 hrs. A cotton swab was used to remove the cells that did not migrate to the other side. 4% paraformaldehyde (Solarbio,#P1110) was used to fix the cells migrating to the lower surface for 20 mins and then hematoxylin (Solarbio,#G1120) was used for staining.

2.6. Scratch assay

Forty-eight hrs post transfection, gently and slowly scratch the monolayer with a new 200 ul pipette tip across the center of the well in the 6-well plate. The wound gap images were taken 0, 24 hrs, 48 hrs after scratch.

2.7. Western blotting

Cells were lysed and protein concentration was measured using a BCA Protein Assay kit (Thermo Scientific,#23227). Cell lysate (30 ug) for each well was managed for conduction of 10% SDS-PAGE. The lysate was then transferred onto the PVDF membrane (Millipore, #IPVH00010) on the basis of the wet transfer for 1 hr. After 1 hr blocking, the membrane was immersed with the primary antibody overnight at 4 °C. Antibodies we used include SUFU(Cell Signaling, (C81H7)#2522), Wnt/β-Catenin Activated Targets Antibody Sampler Kit (Cell Signaling,#8655), including CD44 (156-3C11),#3570; Cyclin D1 (92G2),#2978; c-Jun (60A8),#9165; LEF1 (C12A5),#2230; Met (D1C2),#8198; MMP-7 (D4H5),#3801; c-Myc (D84C12),#5605; TCF1/TCF7(C63D9),#2203; Anti-mouse IgG (#7076); Anti-rabbit IgG (#7074) and Epithelial-Mesenchymal Transition (EMT) Antibody Sampler Kit (1:1000, Cell Signaling,#9782) including Vimentin (D21H3) #5741; N-Cadherin (D4R1H)#13116; Claudin-1 (D5H1D)#13255; β-Catenin (D10A8) #8480; ZO-1 (D7D12)#8193; Snail (C15D3)#3879; Slug (C19G7) #9585; ZEB1 (D80D3)#3396; E-Cadherin (24E10) #3195; Anti-rabbit IgG#7074 and GAPDH antibody (1:5000, Cell Signaling, (D16H11) #5174S). The second day, the membranes were washed three times and incubated with secondary antibodies at room temperature for 1 hr. Chemiluminescence substrates (Thermo Scientific, #1859700) were used for exposure. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control.

2.8. Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde (Solarbio, #P1110) for 15 mins and then permeabilized with 0.2% Triton X-100(Solarbio,#P1080) for 10 mins. After blocking with 1% BSA (Solarbio,#SW3015) for 30 mins, cells were incubated with β-catenin antibody (1:100; Cell Signaling (L54E2)#2677) overnight at 4 °C.The second day, the cells were incubated with secondary antibody (1:1,000, Cell Signaling #8890) for 1 hr. DAPI II was used for nuclei staining (Solarbio, #D8200). Leica SP5 AOBS confocal microscope was used to take photos (Cellular Imaging Facility, Lausanne, Switzerland).

2.9. Colony-formation assay

Cells were replated in a 6-well plate at a density of 200 cells/well 48 hrs after transfection. Two weeks later, 4% paraformaldehyde was used

for fixing cells and hematoxylin (Solarbio,#G1120) was used for staining. The number of cell colonies was counted.

2.10. Luciferase activity assay

To measure the activity of Wnt signaling, Topflash/Foplash assay was performed. Topflash and Fopflash plasmids were purchased from Addgene. Topflash plasmid contains 7 TCF/LEF binding sites while Fopflash plasmid contains 6 mutated TCF/LEF binding sites, which locate in the upstream of the luciferase reporter. 60nM of miRNA inhibitors or mimics as well as Topflash/Fopflash vector together with plasmid pTK-renilla were cotransfected. Forty-eight hrs later, Dual-Glo luciferase assay kit (Promega, #E1910) was used to measure the luminescence intensity.

To further investigate how miRNA-20b activates Wnt signaling, target genes of miR-20b were predicted using miRwalk website (http://mirwalk.umm.uni-heidelberg.de/). SUFU was chosen with high score and involved in Wnt signaling.

For miRNA binding luciferase activity assay, SUFU-3′UTR segments containing putative miRNA-20b binding sites were inserted into pDL-UTR vector (Promega, USA). The corresponding mutated vector (pDL-SUFU-3′UTR-mut) was constructed by Shenzhen AngRan Co., Ltd. Primers for 6nt sequence mutation of binding site were designed. The 3′UTR mutated fragments were amplified from SUFU-3′UTR vector using specific primers. Amplicons were cut by EcoRI and XhoI and cloned into pDL vectors. Sixty nM miRNA and 40 ng of plasmids were cotransfected.

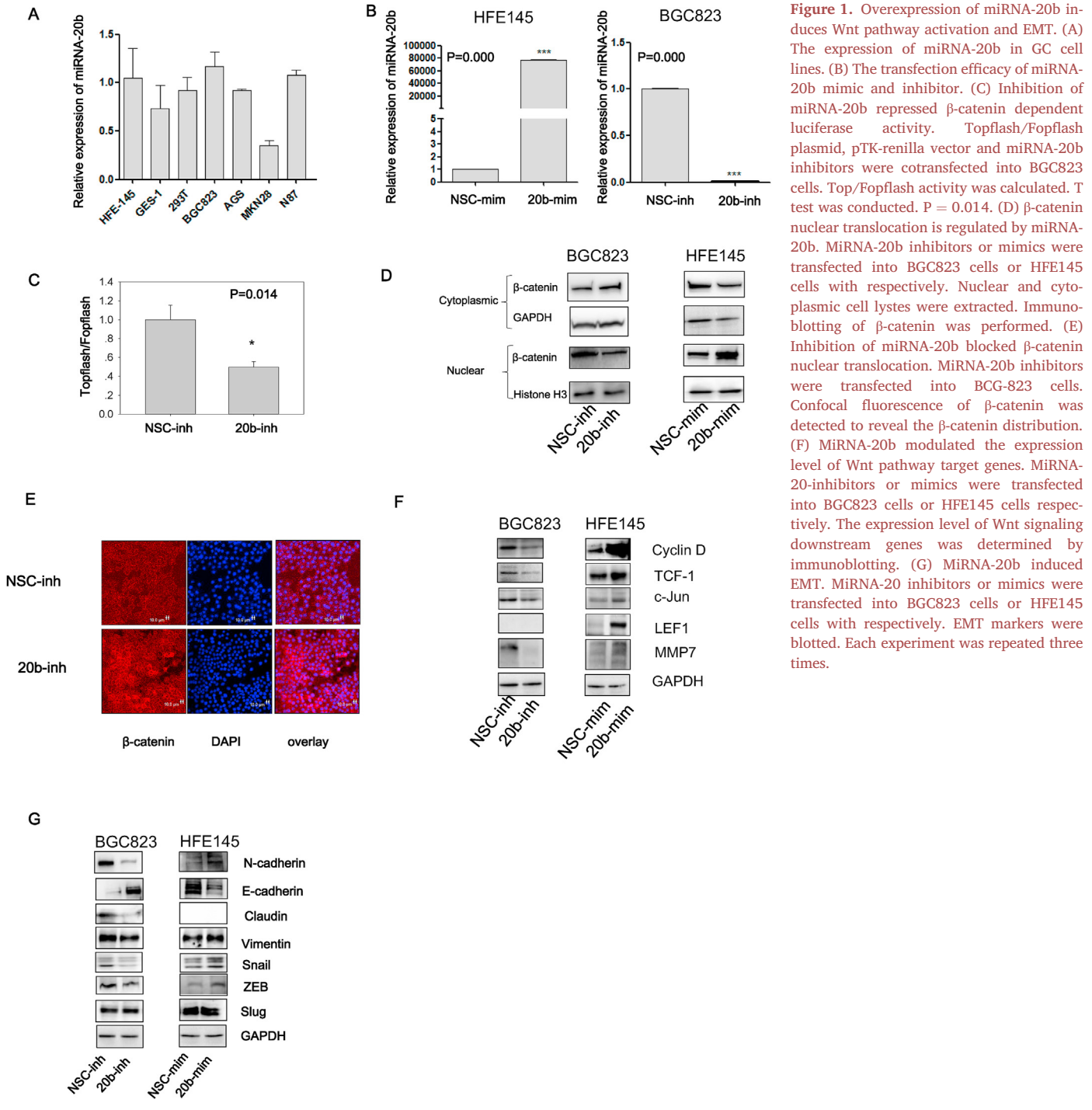

Figure 1. Overexpression of miRNA-20b induces Wnt pathway activation and EMT. (A) The expression of miRNA-20b in GC cell lines. (B) The transfection efficacy of miRNA-20b mimic and inhibitor. (C) Inhibition of miRNA-20b repressed β-catenin dependent luciferase activity. Topflash/Fopflash plasmid, pTK-renilla vector and miRNA-20b inhibitors were cotransfected into BGC823 cells. Top/Fopflash activity was calculated. T test was conducted. P = 0.014. (D) β-catenin nuclear translocation is regulated by miRNA-20b. MiRNA-20b inhibitors or mimics were transfected into BGC823 cells or HFE145 cells with respectively. Nuclear and cytoplasmic cell lystes were extracted. Immunoblotting of β-catenin was performed. (E) Inhibition of miRNA-20b blocked β-catenin nuclear translocation. MiRNA-20b inhibitors were transfected into BCG-823 cells. Confocal fluorescence of β-catenin was detected to reveal the β-catenin distribution. (F) MiRNA-20b modulated the expression level of Wnt pathway target genes. MiRNA-20-inhibitors or mimics were transfected into BGC823 cells or HFE145 cells respectively. The expression level of Wnt signaling downstream genes was determined by immunoblotting. (G) MiRNA-20b induced EMT. MiRNA-20 inhibitors or mimics were transfected into BGC823 cells or HFE145 cells with respectively. EMT markers were blotted. Each experiment was repeated three times.

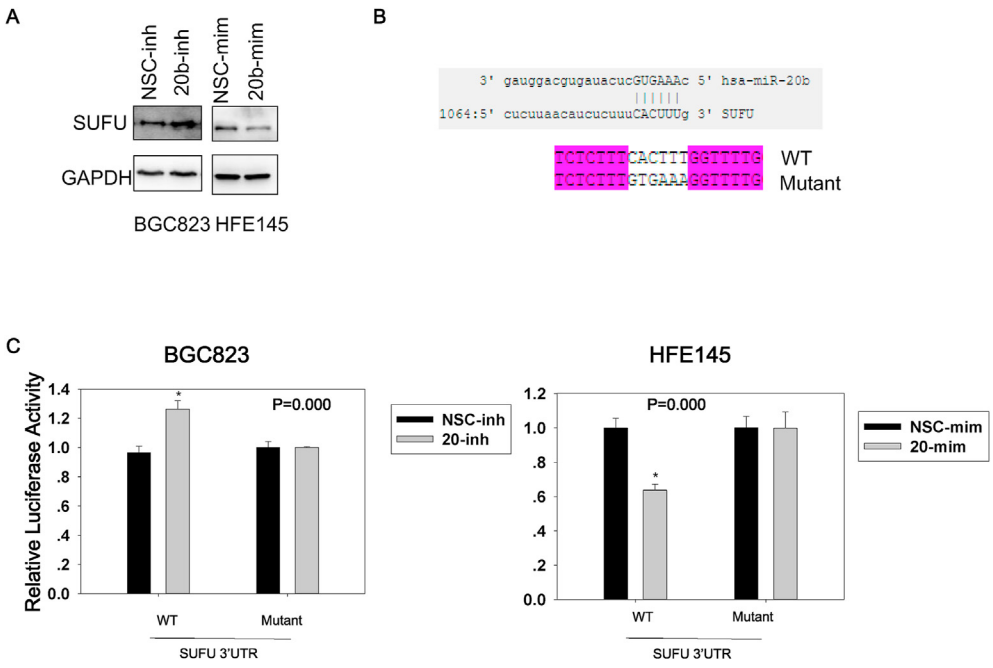

Figure 2. MiRNA-20b targets SUFU. (A) Downregulation of miRNA-20b resulted in elevated expression level of SUFU while overexpression of miRNA-20b mimics leads to reduction of SUFU expression. MiRNA-20b inhibitors or mimics were transfected into BGC823 and HFE145 cells, respectively. Immunoblotting of SUFU was performed. (B) Sequence analysis of SUFU 3′UTR revealed one potential binding site for miRNA-20b. A wild type and a mutant reporter were constructed as shown. (C)Upregulation of miRNA-20b inhibited wild type luciferase reporter activity while downregulation of miRNA-20b elevated the luciferase activity but not the mutant. T test was conducted. P = 0.000, respectively. Each experiment was repeated three times.

Two days later, the relative luciferase activity was determined as described above.

2.11. Statistical analyses

SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) was used in the statistical analyses. T test was applied for analyzing differences between two groups. Comparison analyses in datasets containing multiple groups were performed by using one-way analysis of variances followed by Dunnett's test. All Data are shown as the mean ± SD of the mean. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. MiRNA-20b activates Wnt pathway and EMT

Based on previous research, miRNA-20b is implicated in GC tumorigenesis, however the underlying mechanism remains rudimentary. We first examined the expression level of miRNA-20b in GC cell lines and found that miRNA-20b was expressed at comparable level in gastric cancer cells including BGC823, AGS and N87, and HFE145 which is a normal gastric cell line (Figure 1A). The transfection efficacy of miRNA-20b mimic and miRNA-20b inhibitor has been determined. The expression of miRNA-20b was significant up-regulated in HFE145 with 20b-mimic transfection while down-regulated in BGC823 with 20b-inhibitor transfection (Figure 1B). To determine the relationship between miRNA-20b and Wnt signaling in GC, a Topflash assay was performed. Inhibition of miRNA-20b results in reduced relative TCF dependent transcriptional activity, suggesting that miRNA-20b sustains Wnt signaling (Figure 1C, P = 0.014). Furthermore, we accessed the β-catenin*(CTNNB1)* nuclear and cytoplasmic distribution using subcellular fractionation followed by immunoblotting. Inhibition of miRNA-20b sequesters more β-catenin in the cytoplasm in BGC823 cells, consistently, overexpression of miRNA-20b in HFE145 cells promotes more β-catenin translocate to the nucleus, implying that miRNA-20b activates Wnt signaling pathway (Figure 1D). To confirm the immunoblotting results and view the β-catenin localization morphologically, confocal microscope coupled with immuno-fluorescent staining was used to detect the β-catenin localization change. In agreement with the immunoblotting results, miRNA-20b inhibitors cause β-catenin preferentially localized to cell membrane compared to the control (Figure 1E). In addition, the expression level of Wnt pathway downstream target was investigated. As shown in Figure 1F, inhibition of miRNA-20b consequently downregulated a set of Wnt target genes including cyclin D1(CCND1), TCF-1(*HNF1A*), c-Jun (*JUN*), LEF1 (*LEF1*), MMP7(*MMP7*). Conversely, overexpression of miRNA-20b in HFE145 cells induced the expression of these genes, suggesting that miRNA-20b truly activates the Wnt/β-catenin activity in turn upregulates the downstream gene expression. EMT is a critical process required for invasion and dissemination of carcinoma cells. It involves the dissociation of β-catenin from adherens junctions, the same as Wnt pathway activation. Thus, we explored the expression level of EMT associated markers. It showed that inhibition of miRNA-20b led to a decrease in N-cadherin*(Cdn2)*, Claudin*(CLDN1)*, Vimentin*(VIM)*, EMT transcriptional factor ZEB*(ZEB1)* and Snail*(SNAI1)* and an increase in E-cadherin*(Cdn1)* expression (Figure 1G). On the other hand, enforced overexpression of miRNA-20b resulted in a remarkable enhancement of N-cadherin, Claudin, Vimentin, ZEB and Snail expression and a decrease in E-cadherin expression.

Aberrantly activated Wnt signaling elevates the Snail expression level in CRC (Colorectal Cancer) cells [13]. Snail/Slug*(SNAI2)* are transcriptional repressors binding to the promoter of E-cadherin and inhibiting its expression. Here, we found that miRNA-20b only influences Snail expression but not Slug. It is a good control for expression change of other EMT markers, suggesting that miRNA-20b specifically regulates Snail not Slug to modulate the EMT. Claudin is lowly expressed in HFE145 cells.

3.2. SUFU is identified as a target of miRNA-20b

To further investigate how miRNA-20b activates Wnt signaling, we have integrated miRNA target prediction using miRwalk (http://mirwalk.umm.uni-heidelberg.de/) and analysis of Wnt pathway associated genes. We identified SUFU, negatively regulating Wnt pathway, potentially targeted by miRNA-20b. The prediction was confirmed by the experimental results revealing that transfection of inhibitors of miRNA-20b enhanced the SUFU expression in BGC823 cells and forced overexpression of miRNA-20b mimics decreased the expression of SUFU in HFE145 cells (Figure 2A). Sequence analysis of 3′UTR of SUFU revealed that there is a potential binding site for miRNA-20b. Subsequently, the reporters containing wild type or mutant binding site for miRNA-20b were constructed (Figure 2B). MiRNA-20b suppressed the activity of

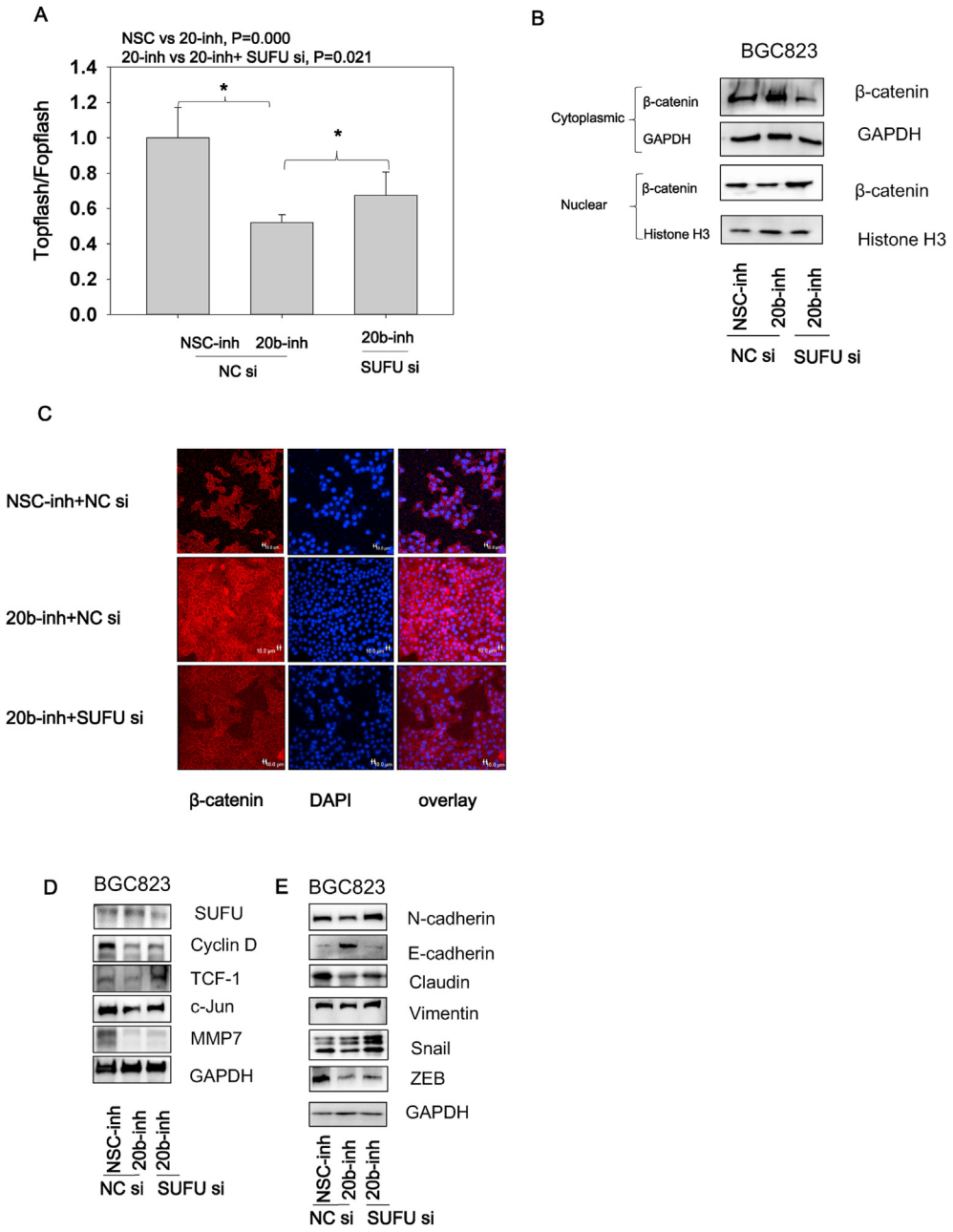

Figure 3. MiRNA-20b/SUFU/Wnt axis induces EMT. (A–E) MiRNA-20b inhibitors or miRNA-20b inhibitors combined with SUFU siRNAs were transfected into BGC823 cells. (A) Suppression of miRNA-20b inhibited β-catenin dependent luciferase activity and knockdown of SUFU partially alleviated the inhibition. NSC vs 20-inh, P = 0.000; 20-inh vs 20-inh + SUFU si, P = 0.021. (B–C) Inhibition of β-catenin nuclear translocation caused by miRNA-20b repression was relieved by reduction of SUFU. Western blotting (B) and confocal immunofluorescence (C) of β-catenin was performed to reveal the β-catenin cytoplasmic or nuclear localization. (D) Wnt signaling inhibition caused by miRNA-20b suppression was rescued by coupled transfection of SUFU siRNAs. The cell lysates were immunoblotted with antibodies of Wnt signaling downstream genes. (E) MiRNA-20b/SUFU/Wnt axis induces EMT. The cell lysates were blotted with antibodies of EMT markers. Each experiment was repeated three times.

the SUFU 3′UTR reporter containing WT (wild type) binding site of miRNA-20b in HFE145 cells (Figure 2C, P = 0.000), but not the mutant reporter. Agreeably, transfection of inhibitors of miRNA-20b elevated the luciferase expression of the reporter containing WT 3′UTR of SUFU in BGC823 cells, but not the mutant reporter (Figure 2C, P = 0.000). It confirms that miRNA-20b binds to 3′UTR of SUFU to target SUFU.

3.3. MiRNA-20b/SUFU/Wnt axis induces EMT

To determine if miRNA-20b activates Wnt pathway and EMT via targeting SUFU, we performed the rescue experiment by knocking down SUFU meanwhile inhibiting miRNA-20b. The reduction of Topflash/Fopflash induced by inhibitors of miRNA-20b was rescued (Figure 3A, NSC vs 20-inh, P = 0.000; 20-inh vs 20-inh + SUFU si, P = 0.022). Inhibition of β-catenin nuclear translocation triggered by miRNA-20b inhibitors was relieved after introducing SUFU siRNAs to miRNA-20b inhibitors as revealed by immuno-blotting after subcellular fractionation (Figure 3B). Consistent with this, as revealed in immunofluorescence results (Figure 3C), miRNA-20b inhibition resulted in more β-catenin preferentially localized to the cellular membrane, however, miRNA-20b inhibitors combined with SUFU siRNAs reversed the translocation. Consequently, the expression reduction of Wnt pathway downstream gene was alleviated by additional transfection of SUFU siRNAs (Figure 3D).

In order to test if EMT induced by miRNA-20b was mediated by SUFU, we detected EMT markers after miRNA-20b inhibition as well as combinational treatment of miRNA-20b inhibitors and SUFU siRNAs. The results showed that SUFU siRNAs promoted EMT which was suppressed by miRNA-20b inhibition, suggesting that SUFU mediating the EMT triggered by miRNA-20b (Figure 3E).

3.4. SUFU is a mediator of miRNA-20b tumorigenic property

MiRNA-20b has been reported as an oncogenic miRNA is GC. We would like to know if SUFU is a mediator of part of the biological function of miRNA-20b. CCK8 data showed that knockdown of SUFU rescued part

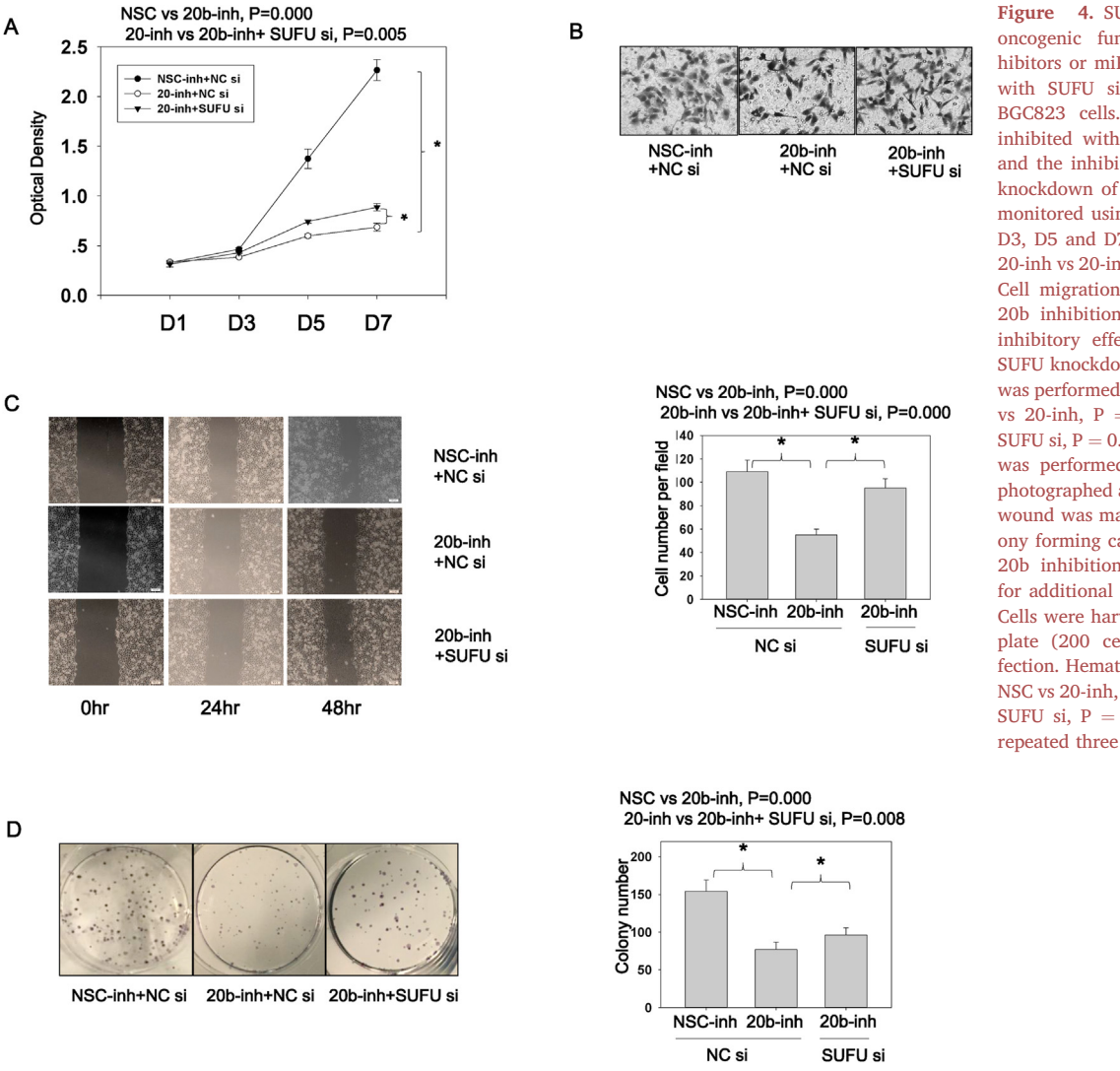

Figure 4. SUFU mediates miRNA-20b oncogenic function. (A-D)MiRNA-20b inhibitors or miRNA-20b inhibitors combined with SUFU siRNAs were transfected into BGC823 cells. (A) Cell proliferation was inhibited with the miRNA-20b knockdown and the inhibition was partially rescued by knockdown of SUFU. Cell proliferation was monitored using CCK8 assay at day 1 (D1), D3, D5 and D7. NSC vs 20-inh, P = 0.000; 20-inh vs 20-inh + SUFU si, P = 0.005. (B–C) Cell migration was impaired with miRNA-20b inhibition in BGC823 cells while the inhibitory effect was relieved by coupled SUFU knockdown. (B) Migration experiment was performed 48 hrs after transfection. NSC vs 20-inh, P = 0.000; 20-inh vs 20-inh + SUFU si, P = 0.000. (C) Wound healing assay was performed. The scratch pictures were photographed at 0 hr, 24 hrs and 48 hrs after wound was made. (D) Reduction of cell colony forming capacity resulted from miRNA-20b inhibition and was reversed partially for additional transfection of SUFU siRNAs. Cells were harvested and seeded into 6-well plate (200 cells/well) 48 hrs post transfection. Hematoxylin was used to stain cells. NSC vs 20-inh, P = 0.000; 20-inh vs 20-inh + SUFU si, P = 0.008. Each experiment was repeated three times.

of the proliferation rate reduction caused by miRNA-20b inhibitors (Figure 4A, NSC vs 20-inh, P = 0.000; 20-inh vs 20-inh + SUFU si, P = 0.005). Cell migration suppressed by miRNA-20b inhibitors was enhanced by SUFU siRNAs as demonstrated in transwell and wound healing assay (Figure 4B, NSC vs 20-inh, P = 0.000; 20-inh vs 20-inh + SUFU si, P = 0.000, and Figure 4C). Cell colony formation capacity was decreased in miRNA-20b inhibitors treated cells and rescued in coupled SUFU siRNAs transfected counterparts (Figure 4D, NSC vs 20-inh, P = 0.000; 20-inh vs 20-inh + SUFU si, P = 0.008). Taken together, it suggests that SUFU is a mediator of the tumorigenic property of miRNA-20b to a certain degree.

4. Discussion

MiRNA-20b is a member of the miRNA-106a-363 clusters, with miRNA-106b-25 and miRNA-17-92 together constitutes miRNA-17 family [14]. MiRNA-20b displays context-dependent tumor relevant properties in regulating oncogenesis of various cancers [15]. MiRNA-20b has been found to be overexpressed in a number of cancers including HCC (hepatocellular carcinoma) [16], GC [3], breast cancer [14, 17], NSCLC (non-small cell lung cancer) [18], prostate cancer [19], CRC [20] and esophageal cancer [21]. Particularly, most studies of miRNA-20b were conducted in breast cancer. It is found that miRNA-20b promotes breast cancer by targeting PTEN (phosphatase and tensin homolog deleted on chromosome ten), a well-recognized tumor suppressor [14]. Similar to breast cancer, miRNA-20b also targets PTEN in a panel of cancers such as prostate cancer [19], HCC [22] and esophageal cancer [21], acting as an oncogene.

Targeting miRNA-20b may have a therapeutic potential. Inhibition of miR-20b reduces tumor volume of breast cancer cells in nude mice [14]. MiRNA-20b is associated with the pathological features including tumor differentiation, invasion depth, lymph node metastasis, TNM stage, and overall survival in esophageal carcinoma [21, 23]. Aberrant expression of miRNA-20b increases tumor growth in *in vivo* study [23], together providing the rationale to target miRNA-20b in esophageal carcinoma. Similarly, there is a significant correlation between miRNA-20b and pathological characteristics in HCC [22]. In addition, previous studies revealed that miRNA-20b expression was enhanced in GC tissue and was positively associated with lymph node metastasis [3]. Further, high level of miRNA-20b predicts poor survival for GC patients [24] and

suppression of miRNA-20b displays an anti-tumor effect *in vitro* [25], suggesting the therapeutic potential of targeting miRNA-20b in GC. However, the oncogenic molecular mechanism of miRNA-20b is not well studied.

SUFU has been found as a tumor suppressor gene in GC. It is a negative regulator of hedgehog signaling pathway by binding to Gli. The expression of SUFU is downregulated in GC [26, 27, 28, 29]. Previous literature has indicated that SUFU was down expressed in GC tissues and cells. In this study, we have demonstrated that miRNA-20b targets SUFU in GC. MiRNA targeting is likely to be a critical explanation accounting for the low expression level of SUFU in tumor. A handful of miRNAs have been found to target SUFU in many cancers. MiR-378 inhibits SUFU to accelerate the glioma cell growth and angiogenesis [30]. MiR-214 enhances EMT and metastasis in lung adenocarcinoma by targeting SUFU [31]. Exosomal miR-423-5p, identified as a biomarker for GC, represses SUFU to advance cancer growth and metastasis [26]. Here, we show that SUFU is also inhibited by miRNA-20b to enhance cell proliferation and EMT in GC. Taken together, SUFU is a critical tumor suppressor and is targeted by multiple miRNAs in cancers.

The relationship between miRNA-20b and Wnt signaling pathway is far from clear. In a study investigating how the crosstalk between miRNA-20b and Wnt activity modulates GBM (glioblastoma) phenotypes, they have identified a consensus TCF4 (Transcription factor 4)-binding site in the promoter of miRNA-20b. Thus, in proneural GBM, Wnt activity is constitutively active and activates transcription of miRNA-20b through TCF4. Meanwhile, miRNA-20b targets FZD6 (Frizzled-6) and APC, negative regulators of Wnt signaling, further enhancing Wnt activity [32]. Here, we also demonstrate that miRNA-20b activates Wnt pathway through suppressing SUFU expression. The findings are consistent with the fact that miRNA-20b generally activates Wnt signaling pathway through modulating Wnt associated negative regulators.

In conclusion, miRNA-20b/SUFU/Wnt axis accelerates cell proliferation, migration and EMT in human GC. For further research, *in vivo* experiment may be a good choice to explore the deeper role of miRNA-20b. Besides, the relationship between miRNA-20b and series of clinical indicators such as tumor metastasis, TNM stage and overall survival is worth exploring.

Declarations

Author contribution statement

Zhe Jin, Xinmin Fan: Conceived and designed the experiments; Wrote the paper.

Yin Peng: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Xiaojing Zhang: Conceived and designed the experiments; Performed the experiments.

Stephen J Meltzer, Song Li: Conceived and designed the experiments.

Shiqi Deng, Yuan Yuan, Xianling Feng, Yulan Cheng, Fan Hu, Yuli Gao, Jieqiong He, Wangchun Chen, Ying Qin, Shutong Zhuang, Na Tang: Performed the experiments.

Yanjie Wei: Analyzed and interpreted the data.

Hassan Ashktorab, Duane Smoot: Contributed reagents, materials, analysis tools or data.

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Data availability statement

No data was used for the research described in the article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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