Cancer Research

Epigenetic Silencing of miR-490-3p Reactivates the Chromatin Remodeler SMARCD1 to Promote *Helicobacter pylori*–Induced Gastric Carcinogenesis

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Abstract

Chromatin remodeling has emerged as a hallmark of gastric cancer, but the regulation of chromatin regulators other than genetic change is unknown. *Helicobacter pylori* causes epigenetic dysregulation to promote gastric carcinogenesis, but the roles and functions of microRNAs (miRNA) in this multistage cascade are not fully explored. In this study, miRNA expression in preneoplastic and neoplastic lesions in murine stomachs induced by *H. pylori* and *N*-methyl-*N*-nitrosourea (MNU) was profiled by miRNA expression array. miR-490-3p exhibited progressive downregulation in gastritis, intestinal metaplasia, and adenocarcinoma during *H. pylori* and MNU-induced gastric carcinogenesis. Significant downregulation of miR-490-3p was confirmed in human gastric cancer tissues in which its regulatory region was found to be hypermethylated. miR-490-3p exerted growth- and metastasis-suppressive effects on gastric cancer cells through directly targeting SMARCD1, a SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeling complex subunit. Knockdown of SMARCD1 significantly attenuated the protumorigenic effects of miR-490-3p inhibitor, whereas enforced expression of SMARCD1 promoted *in vitro* and *in vivo* oncogenic phenotypes of gastric cancer cells. SMARCD1 was markedly upregulated in gastric cancer in which its high expression was associated with shortened patients' survival independent of TNM staging. In conclusion, hypermethylation-mediated silencing of miR-490-3p reactivates SMARCD1 to confer malignant phenotypes, mechanistically linking *H. pylori*, chromatin remodeling, and gastric carcinogenesis. *Cancer Res; 75(4); 754–65. ©2014 AACR.*

Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death in the world (1). Because of its absence of specific symptoms for early diagnosis, gastric cancer is often diagnosed at an advanced stage with a median survival of about 7 to 9 months (2). *Helicobacter pylori* infection, one of the most prevalent infectious diseases in the world, is associated with both subtypes of gastric cancer, namely the intestinal type and the diffuse type (3). According to the International Agency for Research on Cancer of the World Health Organization, *H. pylori* is classified as a definite (type I) carcinogen, which is now thought to account for more than 80% of gastric cancers. The molecular mechanism underlying the association between *H. pylori* infection and gastric cancer, however, is still not completely understood.

MicroRNAs (miRNA) are a class of short noncoding RNAs (19–24 nucleotides in length) that regulate gene expression negatively. More than 2,500 mature miRNAs have been discovered in the human genome. Functionally, miRNAs carry out biologic effects through direct binding to 3′ untranslated regions (UTR) of their target mRNAs, and thereby inducing mRNA degradation and/or translational repression. Some miRNAs, including miR-21, miR-27a, miR-130b, miR-155, miR-218, and miR-221-222 clusters, have shown differential expression in *H. pylori*–infected mucosa and gastric cancer tissues. Genes coding for these miRNAs are putative proto-oncogenes and tumor-suppressor genes based on findings that these miRNAs control malignant phenotypes of gastric cancer cells by deregulating

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-14-1301

intracellular signaling pathways (4). For instance, miR-218 downregulation contributes to the activation of NF-κB signaling, whereas miR-130b upregulation leads to reduced expression of the tumor-suppressor protein RUNX3 (5, 6). Elucidating the biologic aspects of miRNA dysregulation may help us better understand the pathogenesis of gastric cancer and promote the development of miRNA-directed diagnostics and therapeutics. However, a comprehensive picture depicting miRNA dysregulation during gastric tumorigenesis is still lacking.

In the study of the molecular pathogenesis of gastric cancer, novel discovery has very often been confounded by genetic and pathologic heterogeneities of clinical specimens. Animal models of gastric cancer, thus, represent reliable alternatives. Mice are inbred, thus permitting host variables to be carefully controlled. In this regard, *H. pylori* inoculation together with administration of *N*-methyl-*N*-nitrosourea (MNU) in drinking water has been shown to reproducibly induce preneoplastic and neoplastic lesions in murine stomachs (7). Using an integrative epigenomics approach, we have recently identified concomitant hypermethylated genes in mice infected with *H. pylori* and human gastric cancer samples (8). In this study, we profiled miRNA dysregulation in gastric tissues derived from the *H. pylori* and MNU-induced murine model of gastric cancer by genome-wide miRNA expression array, followed by validation of specific miRNA dysregulation in clinical specimens of human gastric cancer. Using this approach, we successfully identified miR-490-3p as a frequently downregulated miRNA in human gastric cancer. Subsequent analyses further established the tumor-suppressive functions and mechanism of action of this miRNA.

Materials and Methods

Mouse model of gastric cancer

C57BL/6 mice (6–8-weeks-old) were inoculated with *H. pylori* (SS1 strain) without or with MNU. Two hundred and forty parts per million (ppm) MNU was given in drinking water starting from 7 week of age for a total five cycles of a 1-week regimen followed by a 1-week pause. Mice were inoculated with *H. pylori* (1×10^9 colony-forming U/mL) every other day for totally three times starting from 18 weeks of age.

Clinical specimens

Tissues were retrieved from the tissue bank of the Prince of Wales Hospital (Hong Kong). Use of these tissues had been approved by the Joint Chinese University of Hong Kong—New Territories East Cluster Clinical Research Ethics Committee.

Cell lines and transfection

Normal gastric epithelial cell line HFE-145 and gastric cancer cell lines AGS and TMK-1 were used in this study. All cell lines were authenticated with short tandem repeat profiling by the vendor. Transient transfection of miR-490-3p mimics, inhibitor or control oligos was mediated by Lipofectamine 2000. Stable transfection was achieved by lentiviral vectors. See the Supplementary Materials and Methods for complete information.

RNA extraction, expression microarray, and real-time PCR

Total RNA was isolated using TRIzol reagent (Life Technologies). MiRNA and mRNA expression in murine stomachs and human cell lines were profiled using Agilent mouse whole-genome miRNA array (rel15; Agilent Technologies), and the Whole Human Genome Microarray Kit (4×44K; Agilent Technologies), respectively. The microarray data were submitted to Gene Expression Omnibus and assigned accession numbers GSE62503 (miRNA array) and GSE62453 (mRNA array). Reverse transcription and real-time PCR measurement of miRNA were performed using the MicroRNA Reverse Transcription Kit and TaqMan MicroRNA Assays (Applied Biosystems), respectively. U6 was used as an endogenous control.

Methylation-specific PCR and ChIP–qPCR

Genomic DNA was subject to bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research). Bisulfite-treated DNA was then subject to methylation-specific PCR (MSP) using primers specific to methylated or unmethylated *CHRM2/miR-490-3p* promoter as previously described (9). Chromatin immunoprecipitation–quantitative PCR (ChIP–qPCR) for determining the enrichment of histone H3 lysine 4 trimethylation (H3K4me3) was performed using a protocol similar to that of our previous report (8).

Luciferase reporter assay

The wild-type or miR-490-3p–binding site–deleted SMARCD1 3′UTR was subcloned in the pMIR–REPORT reporter (Life Technologies). The pMIR reporter, (200 ng) together with 1 ng of the pRenilla reporter (Promega) were cotransfected into the cells. After 48 hours, transfected cells were retransfected with miR-490-3p mimics or control oligo and incubated for 3 days. The activities of firefly luciferase and *Renilla* luciferase were measured by the Dual-Luciferase Reporter Assay System (Promega).

In vitro and *in vivo* functional assays

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), 5-bromo-2′-deoxyuridine (BrdUrd) incorporation, colony formation, and anchorage-independent cell growth assays were used to assess cell proliferation and/or transformation. Flow cytometry was used to measure cell-cycle distribution. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and Annexin V–propidium iodide staining were used to assess apoptosis. Necrosis was measured by the cellular release of lactate dehydrogenase. Cell migration and invasion were measured with Transwell insert chambers and Matrigel invasion chamber, respectively. *In vivo* tumorigenicity was assessed with a gastric cancer xenograft model in nude mice. See the Supplementary Materials and Methods for complete information.

Western blots and immunohistochemistry

Western blots and IHC were performed following the standard laboratory protocol as previously reported (8). See the Supplementary Materials and Methods for complete information.

Statistical analysis

All data are expressed as mean ± SD from three separate experiments performed in triplicate. Statistical analysis was performed with an ANOVA followed by the Tukey *t* test or Pearson correlation analysis. Relative risks of death were estimated from univariate and multivariate Cox proportional hazards models. Overall survival (OS) in relation to SMARCD1 staining was evaluated by the Kaplan–Meier survival curve and the log-rank test. *P* values less than 0.05 were considered statistically significant.

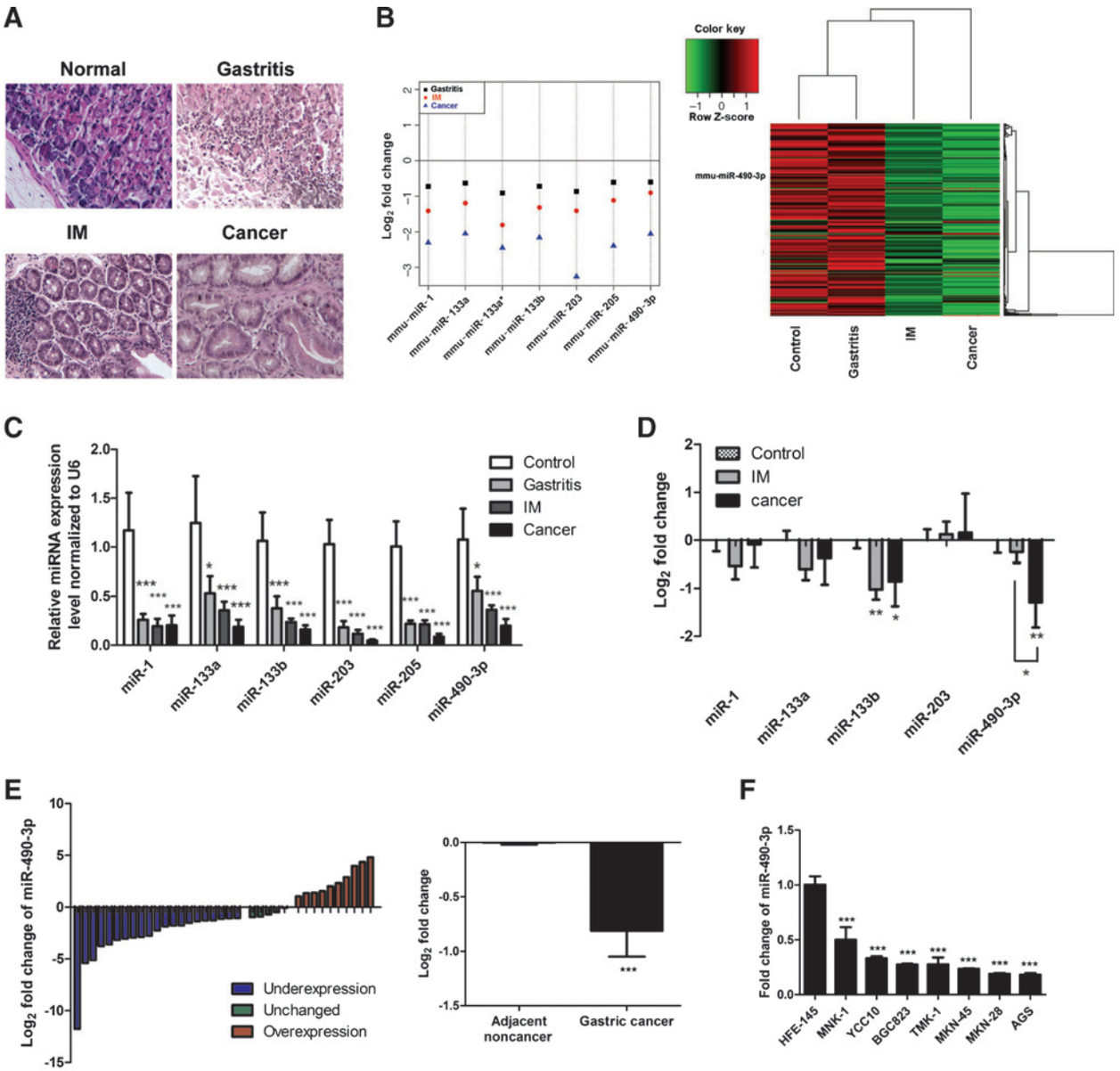


Figure 1

miRNA profiling and validation in murine and human gastric tissues. A, representative H&E-stained tissue sections of normal gastric, chronic gastritis, IM, and gastric adenocarcinoma tissues are shown. B, fold change and heatmap analysis were carried out for miRNA array. C, the downregulation of six candidate miRNAs in chronic gastritis, IM, and gastric adenocarcinoma tissues was verified by real-time PCR. The expression of miR-133a* was not validated because of its lack of functional ortholog in humans. D, the expression of five candidate miRNAs was determined in unpaired normal gastric ($n = 15$), IM ($n = 15$), and gastric adenocarcinoma tissues ($n = 14$) by real-time PCR. miR-205 dysregulation was not verified as its dysregulation in human gastric cancer has been reported. E, expression of miR-490-3p was determined in additional 36 pairs of gastric cancer and tumor-adjacent tissues. About 58% gastric cancer tissues showed >2.0-fold downregulation of miR-490-3p as compared with corresponding tumor-adjacent tissues. F, miR-490-3p expression in all seven gastric cancer cell lines was lower than that of normal gastric epithelial cells HFE-145; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ significantly different from the respective control group or between indicated groups.

Results

H. pylori plus MNU induced intestinal metaplasia and gastric adenocarcinoma in mice

After 12-month carcinogenic regimen, mice were sacrificed and the same part of stomach of each mouse was subject to histologic assessment (Fig. 1A) by two pathologists blinded to experimental groups. Four of 5 mice in the control group had normal histology and the other exhibited very weak inflammation. In mice inoculated with *H. pylori* alone, 3 of 7 (42.9%) exhibited moderate to severe inflammation without preneoplastic or neoplastic changes. In the MNU-plus-*H. pylori* group, 9 of 19 (47.4%) mice developed preneoplastic or neoplastic lesions, namely 4 mice (21.1%) with intestinal metaplasia (IM), 1 (5.3%) with dysplasia, and 4 (21.1%) with gastric adenocarcinoma.

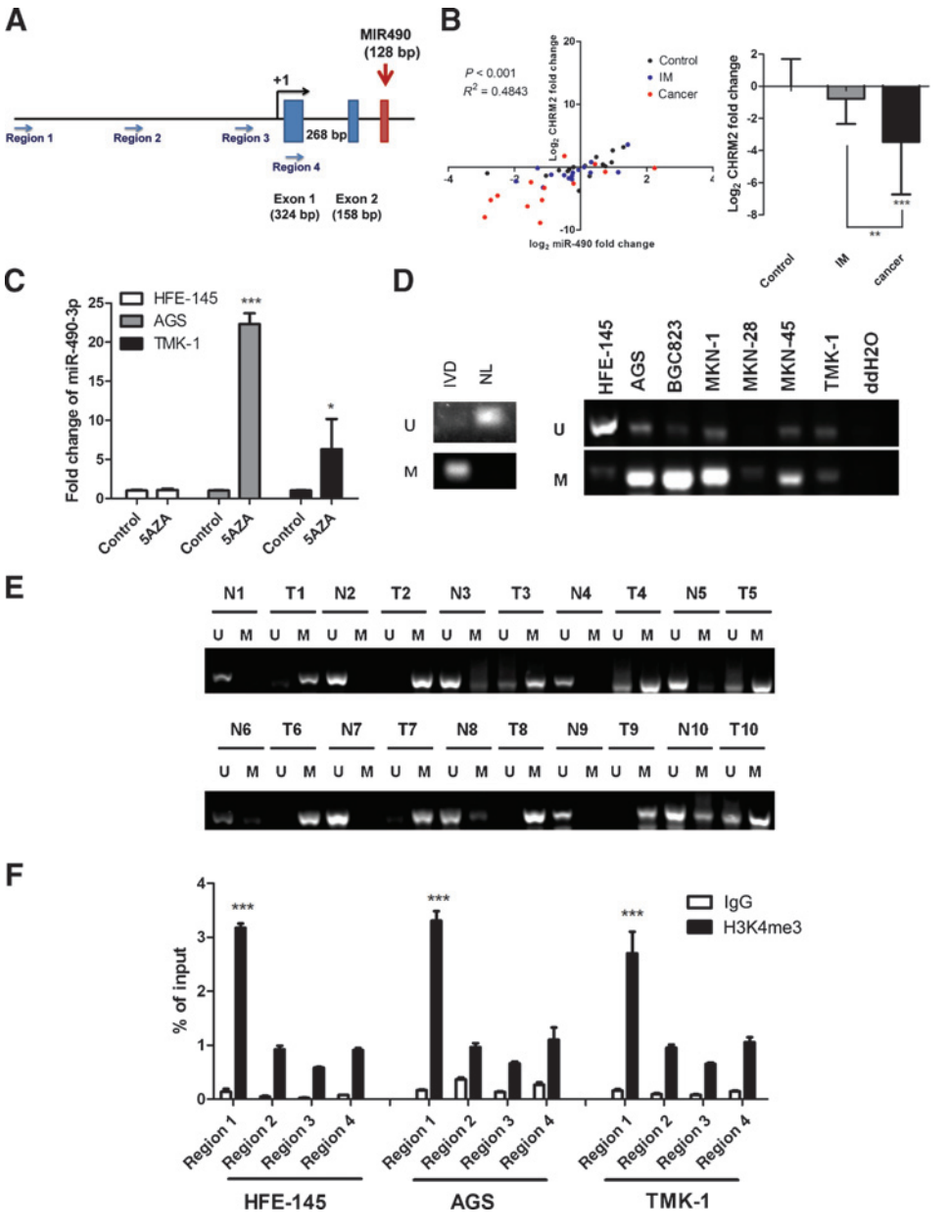


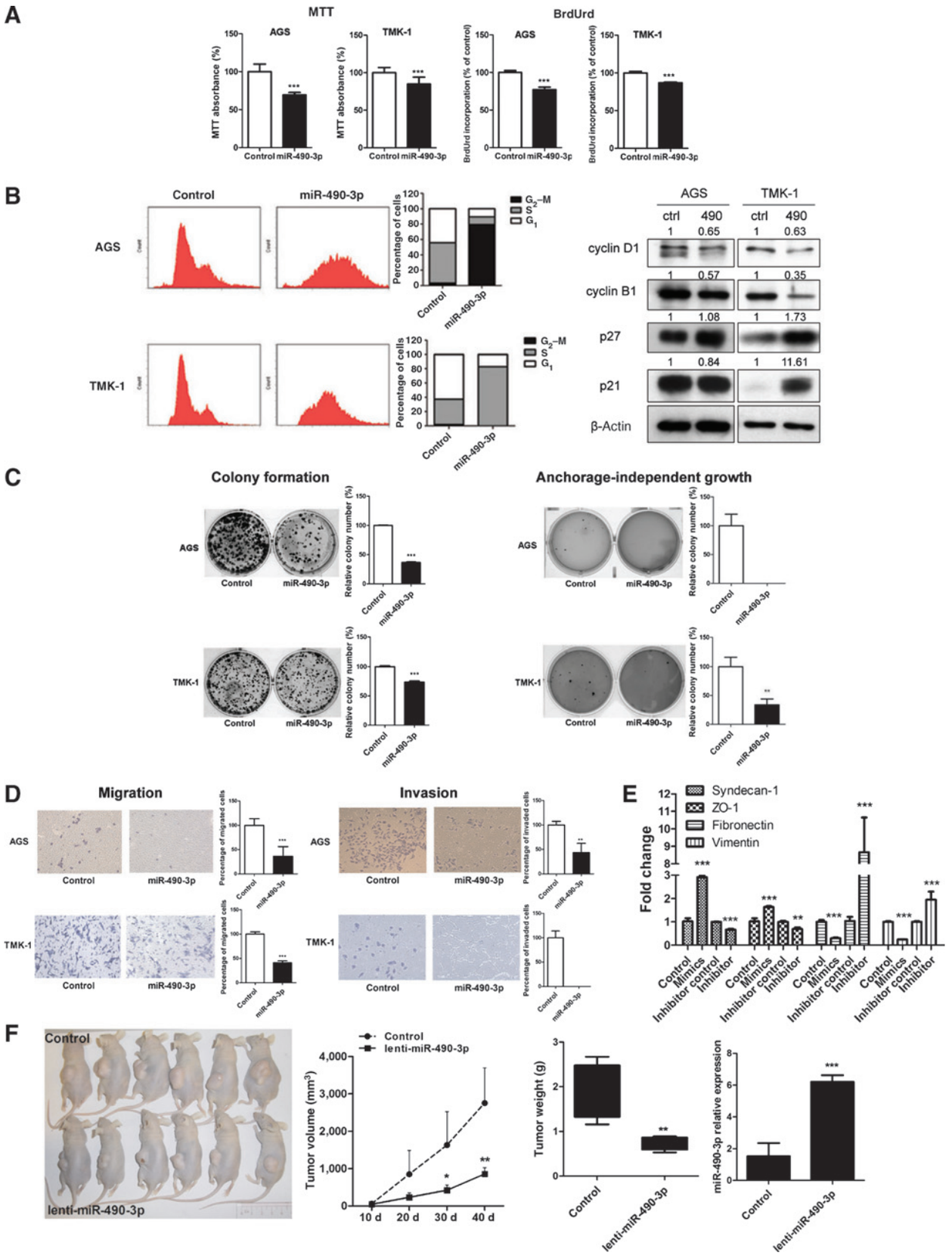
Figure 2
Upstream mechanism of miR-490-3p downregulation in gastric cancer. A, miR-490-3p is located in the second intron of *CHRM2*. B, miR-490-3p levels showed a strong positive correlation with Chrm2 mRNA expression in unpaired human gastric tissues (n = 44). C, treatment of AGS and TMK-1, but not HFE-145 with the DNA-demethylating agent 5-aza-2′deoxycytidine (5AZA; 1 μmol/L, 72 hours) restored miR-490-3p expression. D, hypermethylation of the *miR-490-3p/CHRM2* promoter was detected in six human gastric cancer cell lines as compared with normal gastric epithelial cells HFE-145. U, unmethylated DNA; M, methylated DNA. *In vitro* methylated DNA (IVD) and DNA isolated from normal lymphocyte (NL) were used as positive and negative controls for MSP. E, DNA methylation of the *miR-490-3p/CHRM2* promoter was determined by MSP in 10 pairs of gastric cancer tissues (T1–T10) and tumor-adjacent tissues (N1–N10). All 10 pairs showed increased levels of methylated DNA and reduced levels of unmethylated DNA in gastric cancer tissues versus noncancerous tumor-adjacent tissues. F, the region for MSP analysis (region 1) showed higher enrichment of H3K4me3 as compared with other regions as demonstrated by ChIP–qPCR; *, $P < 0.05$; and ***, $P < 0.001$ significantly different from the respective control group.

microRNA array analysis and real-time PCR validation

Global miRNA expression patterns in inflamed gastric tissues, IM, and gastric adenocarcinoma versus normal gastric tissues obtained from the mouse model were illustrated in Fig. 1B. Seven miRNAs (i.e., mmu-miR-1, mmu-miR-133a, mmu-miR-133a*, mmu-miR-133b, mmu-miR-203, mmu-miR-205, and mmu-miR-490-3p) exhibited absolute $\log_2$ fold change > 2.0 in the gastric cancer tissues. These miRNA also showed progressive downregulation in the inflammation–IM–adenocarcinoma sequence. Hierarchical clustering showed similar progression pattern (Fig. 1B). Validation with real-time PCR confirmed the significant downregulation of miR-133a, miR-133b, miR-203, and miR-490-3p during gastric tumorigenesis (Fig. 1C).

miR-490-3p was downregulated in human gastric cancer

In unpaired clinical samples of human gastric cancer, significant downregulation of miR-133b and miR-490-3p was confirmed by qPCR. MiR-1 and miR-133a also showed marginal reduction (Fig. 1D). MiR-133b has been reported to be a tumor-suppressing miRNA in gastric cancer previously (10, 11). Therefore, miR-490-3p was chosen for further study. The expression of miR-490-3p was next determined in paired human gastric tissue samples. The expression of miR-490-3p was significantly lower in gastric cancer tissues as compared with corresponding adjacent noncancerous tissue in which approximately 58.3% (21 of 36 pairs) patients with gastric cancer exhibited >2.0-fold downregulation (Fig. 1E). The absolute expression levels of miR-490-3p in noncancerous gastric tissues were comparable with that of miR-



375, a tumor-suppressive miRNA in gastric cancer (12), and let-7b, a downregulated miRNA in *H. pylori*–infected gastric mucosa (Supplementary Table S1; ref. 4). Concordantly, miR-490-3p expression levels were significantly lower in all gastric cancer cell lines when compared with the normal gastric epithelial cells HFE-145 (Fig. 1F).

miR-490-3p gene promoter was hypermethylated in gastric cancer

We next investigated the upstream mechanism of miR-490-3p downregulation in gastric cancer. As miR-490-3p is located in the intronic region of *CHRM2* (cholinergic receptor, muscarinic 2; Fig. 2A), we hypothesized that they might share the same promoter. Consistent with our hypothesis, Chrm2 mRNA levels showed significant downregulation in gastric cancer and exhibited positive correlation with miR-490-3p levels, supporting their coexpression (Fig. 2B). Treatment with 5-aza-2′deoxycytidine (a DNA-demethylating agent) restored miR-490-3p expression in AGS and TMK-1, but not HFE-145 (Fig. 2C), suggesting the possible involvement of promoter hypermethylation. Using MSP, we found that hypermethylation of the *CHRM2/miR-490-3p* promoter was observed in human gastric cancer cell lines as compared with normal gastric epithelial HFE-145 cells (Fig. 2D). DNA methylation levels in the promoter of *CHRM2/miR-490-3p* were also substantially higher in human gastric cancer tissues compared with corresponding noncancer adjacent tissues (Fig. 2E). To ascertain whether the region we selected for MSP was the promoter of CHRM2/miR-490-3p, ChIP–qPCR was performed to determine H3K4me3 enrichment in *CHRM2/miR-490-3p* upstream sequence. H3K4me3 has been reported as a reliable marker of the microRNA promoter (13, 14). Only the MSP region, but not other nearby CpG islands showed enrichment of H3K4me3 (Fig. 2F).

miR-490 inhibited cell proliferation, colony formation, anchorage-independent growth, cell migration, and invasiveness

MTT and BrdUrd incorporation assays showed that the proliferation of AGS and TMK-1 was significantly inhibited by transfection with miR-490-3p mimics (Fig. 3A) without induction of necrotic or apoptotic cell death (Supplementary Fig. S1). Cell-cycle analysis revealed that miR-490-3p induced G_2–M phase arrest in AGS and intra-S phase arrest in TMK-1 (Fig. 3B). Consistent with its cytostatic effects, downregulation of cyclin B1 and cyclin D1 was observed in miR-490-3p mimics-transfected AGS and TMK-1. In TMK-1, upregulation of cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ was also noted (Fig. 3B). Moreover, miR-490-3p inhibited the colony-forming abilities (Fig. 3C), anchorage-independent growth (Fig. 3C), cell migration, and invasiveness (Fig. 3D) of both AGS and TMK-1. As miR-490-3p affected cell migration and invasion, the mRNA expression of epithelial–mesenchymal transition (EMT) markers was determined. Consistent with the phenotype, miR-490-3p upregulated the epithelial markers (i.e., syndecan-1 and ZO-1) and downregulated the mesenchymal markers (i.e., fibronectin and vimentin) whereas miR-490-3p inhibitor exerted opposite effects (Fig. 3E). qPCR confirmed that miR-490-3p levels in mimics-transfected cells were comparable with that of noncancerous gastric mucosa (Supplementary Fig. S2A), indicating that the functional effects observed were physiologically relevant.

Stable expression of miR-490-3p inhibited the growth of gastric cancer xenograft in nude mice

We next determined the effect of miR-490-3p on the growth of gastric cancer xenografts in nude mice. Lentivirus-mediated stable overexpression of miR-490-3p significantly inhibited tumor size and tumor weight of TMK-1 (Fig. 3F) and MKN-45 xenografts (Supplementary Fig. S2B) as compared with respective control groups transduced with empty lentiviral particles. At day 40 after inoculation, miR-490-3p still showed significantly higher levels in TMK-1 xenografts of the stable overexpression group (Fig. 3F).

SMARCD1 was the direct target of miR-490-3p

To identify the direct target of miR-490-3p, an integrative approach combining *in silico* prediction, whole-genome mRNA expression array and luciferase reporter assay was used. As shown in Fig. 4A, SMARCD1 and PCBP2 were identified as putative targets of miR-490-3p by miRWalk (at least 4 predicting programs) and DIANA (miTG score > 0.9), and found to be downregulated (fold change >2.0 and $P < 0.05$) in AGS and TMK-1 upon transfection with miR-490-3p mimics as determined by expression array. Measurement of SMARCD1 and PCBP2 mRNA expression by qPCR revealed that SMARCD1 but not PCBP2 was upregulated in human gastric cancer cell lines (Supplementary Fig. S3), indicating that SMARCD1 might be the direct oncotarget of miR-490-3p. qPCR and Western blots further confirmed the downregulation of SMARCD1 by miR-490-3p at both mRNA (Fig. 4B) and protein levels (Fig. 4C), respectively, in both AGS and TMK-1. In contrast, miR-490-3p inhibitor restored SMARCD1 expression (Fig. 4C). To ascertain the direct physical interactions between miR-490-3p and SMARCD1 3′UTR, dual luciferase assay was performed in AGS and HEK293T cells using constructs containing the putative binding site downstream of the reporter gene. As shown in Fig. 4D, miR-490-3p reduced the SMARCD1 3′UTR

Figure 3

In vitro and *in vivo* tumor-suppressive effects of miR-490-3p. A, transfection of miR-490-3p mimics into AGS and TMK-1 reduced cell proliferation as revealed by MTT and BrdUrd incorporation assays (72 hours posttransfection). B, DNA histograms showed the induction of G_2–M and intra-S phase cell-cycle arrest by miR-490-3p mimics in AGS and TMK-1, respectively (72 hours posttransfection). Cells were incubated in serum-free medium for 24 hours followed by 24 hours of serum-replete medium before fixation. miR-490-3p mimics also altered the expression of key cell-cycle regulators in AGS and TMK-1. C and D, miR-490-3p mimics impaired the colony-forming ability (2 weeks post-transfection), anchorage-independent growth (2 weeks posttransfection; C), cell migration (48 hours posttransfection), and cell invasion (72 hours posttransfection) of AGS and TMK-1 (D). E, mRNA expression of epithelial (i.e., syndecan-1 and ZO-1) and mesenchymal (i.e., fibronectin and vimentin) markers was determined in AGS transfected with miR-490-3p mimic or inhibitor. F, inhibition of the growth of gastric cancer xenografts in nude mice by lentiviral transduction of miR-490-3p. Nude mice were inoculated with lentivirus-transduced TMK-1 cells with or without overexpression of miR-490-3p and sacrificed 40 days after inoculation. The size of tumors arising from TMK-1 stably expressing miR-490-3p was significantly lower than those of the control group. Tumor weight was measured at the time of tissue harvesting and showed significant reduction in miR-490-3p–overexpessing xenografts; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ significantly different from the respective control group.

luciferase activity in both AGS and HEK293T. Importantly, the repression of luciferase activity was abrogated when the wild-type binding site was deleted. The protein expression of SMARCD1 as determined by IHC also showed significant downregulation in TMK-1 xenografts overexpressing miR-490-3p (Fig. 4E).

Functional involvement of SMARCD1 in tumor-suppressing effects of miR-490-3p

To determine the functional involvement of SMARCD1, the effect of SMARCD1 knockdown was determined. SMARCD1 knockdown phenocopied the effects of miR-490-3p by inhibiting colony formation (Supplementary Fig. S4A), cell migration (Supplementary Fig. S4B), and invasiveness (Supplementary Fig. S4C) of TMK-1 and AGS. More importantly, SMARCD1 knockdown weakened the promoting effects of miR-490-3p inhibitor on various oncogenic phenotypes, namely cell proliferation (Fig. 5A), colony formation (Fig. 5B), cell migration (Fig. 5C), and invasiveness (Fig. 5D), of AGS and TMK-1. Consistently, overexpression of SMARCD1 increased colony formation (Supplementary Fig. S4D), migration (Supplementary Fig. S4E) and *in vivo* tumorigenicity (Supplementary Fig. S4F) of gastric cancer cells. Cotransfecting miR-490-3p and an SMARCD1 construct without 3′UTR into AGS and TMK-1 demonstrated the rescuing effect of SMARCD1 overexpression on miR-490-3p–mediated inhibition of colony formation (Supplementary Fig. S5A) and cell migration (Supplementary Fig. S5B).

SMARCD1 was overexpressed in human gastric cancer tissues and correlated with poorer patients' survival

Tissue expression of SMARCD1 was determined by IHC (Fig. 6A) in unpaired normal and gastric cancer tissues on tissue microarray (Supplementary Table S2 for patients' clinicopathologic features). The specificity of SMARCD1 antibody was determined by performing IHC using cell blocks containing control and SMARCD1 siRNA–transfected AGS cells. Knockdown of SMARCD1 decreased the staining intensity with the antibody (Fig. 6B). SMARCD1 immunoreactivity was not detected in normal gastric mucosa whereas both cytoplasmic and nuclear staining of SMARCD1 could be observed in gastric cancer tissues (Fig. 6C). Importantly, high expression of SMARCD1 was correlated with shortened OS in patients with gastric cancer (Fig. 6D). In univariate Cox regression analysis, high SMARCD1 expression was associated with a significantly increased risk of death. Age, tumor–node–metastasis (TNM) stage and lymph node metastasis were also significant predictors of OS. After the adjustment for potential confounding factors, high SMARCD1 expression, age, and TNM stage but not lymph node metastasis still predicted poorer survival in the multivariate model (Table 1).

Discussion

Chromatin remodeling has emerged as a hallmark of gastric cancer, but the regulation of chromatin regulators other than genetic change is unknown (15–17). Our findings revealed that epigenetic silencing of *miR-490-3p* promoted gastric tumorigenesis by upregulating SMARCD1, a member of the SWItch/Sucrose NonFermentable (SWI/SNF) chromatin remodeling family involved in cancer development (18). MiR-490-3p has been shown to exert tumor-suppressing actions in other types of cancer, such as colon and lung cancers (19, 20). Here, we demonstrated that promoter DNA hypermethylation of its host gene *CHRM2* might account for the downregulation of *miR-490-3p* in gastric cancer. Similar to our study, Ando and colleagues (21) reported that *miR-124a* is silenced by aberrant DNA methylation in gastric cancer as well as *H. pylori*–infected, noncancerous gastric epithelium, favoring the field cancerization model. The mechanisms by which *H. pylori* promotes DNA hypermethylation of tumor-suppressor genes in gastric cancer are not entirely clear, but chronic inflammation induced by *H. pylori* infection, which triggers infiltration of monocytes and production of reactive oxygen and nitrogen species, appears to be involved in methylation induction (22, 23). In this regard, it would be intriguing to explore whether such epigenetic dysregulation of miRNAs might contribute to chromatin remodeling in the development of other malignancies that arise in the context of microbially initiated inflammatory states (24).

Aside from DNA hypermethylation, we observed that treatment with trichostatin A (a histone deacetylase inhibitor) could restore *miR-490-3p* expression in TMK-1 (data not shown), which exhibited similar levels of methylation and unmethylation in the *miR-490-3p*/*CHRM2* promoter, suggesting that histone deacetylation may also play a part in aberrant silencing of *miR-490-3p*/*CHRM2* in some circumstances. H3K4me3 has been widely regarded as a histone mark for gene promoter (13, 14). Indeed the MSP region showed preferential enrichment of H3K4me3, indicating that this region functions as an important regulatory sequence for *CHRM2*/*miR-490-3p*. It is noteworthy that the normal and two cancerous gastric epithelial cell lines displayed similar levels of H3K4me3 in this region despite substantial DNA hypermethylation in the latter. Although mutual exclusivity between DNA methylation and H3K4me3 has been shown in pluripotent and differentiated cells (25), high level of H3K4me3 can coexist with DNA hypermethylation, particularly in regulatory regions outside of core promoters (26). Consistently, CHRM2 also showed significant downregulation in gastric cancer. Although its function in tumorigenesis remains unknown, other members of the muscarinic cholinergic receptor family have been reported to exert oncogenic or tumor-suppressing functions depending on biologic context (27, 28).

SMARCD1 (also known as BAF60a) has been shown to interact with a wide repertoire of transcription factors, including Oct3/4, Sox2, Sox10, c-Fos/c-Jun, peroxisome proliferator–activated receptor α, vitamin D receptor, glucocorticoid receptor, retinoid-related orphan receptor α, and androgen receptor, to modulate gene expression (29–37). To this end, SMARCD1 is required for maintaining the pluripotency of embryonic stem cells and Tbx1-driven expression of Wnt5a, a noncanonical Wnt ligand that promotes cell migration and invasion in gastric cancer (38–40). In prostate cancer, SMARCD1 is the direct target of the tumor-suppressive miR-99 family (41). A recent study further demonstrated that posttranscriptional downregulation of SMARCD1 by miR-100 could inhibit self-renewal of cancer stem-like cells (42). Nevertheless, the tumor-suppressive properties of SMARCD1 have also been reported. For instance, the interaction between SMARCD1 and p53 is required for p53-mediated cell-cycle arrest and apoptosis (43). SMARCD1 is also frequently inactivated by truncating mutations in breast cancer (44). Such findings indicate a complex and context-dependent function of SMARCD1 in carcinogenesis. We herein found that SMARCD1 downregulation, at least in part, mediates the tumor-suppressive action of miR-490-3p in gastric cancer.

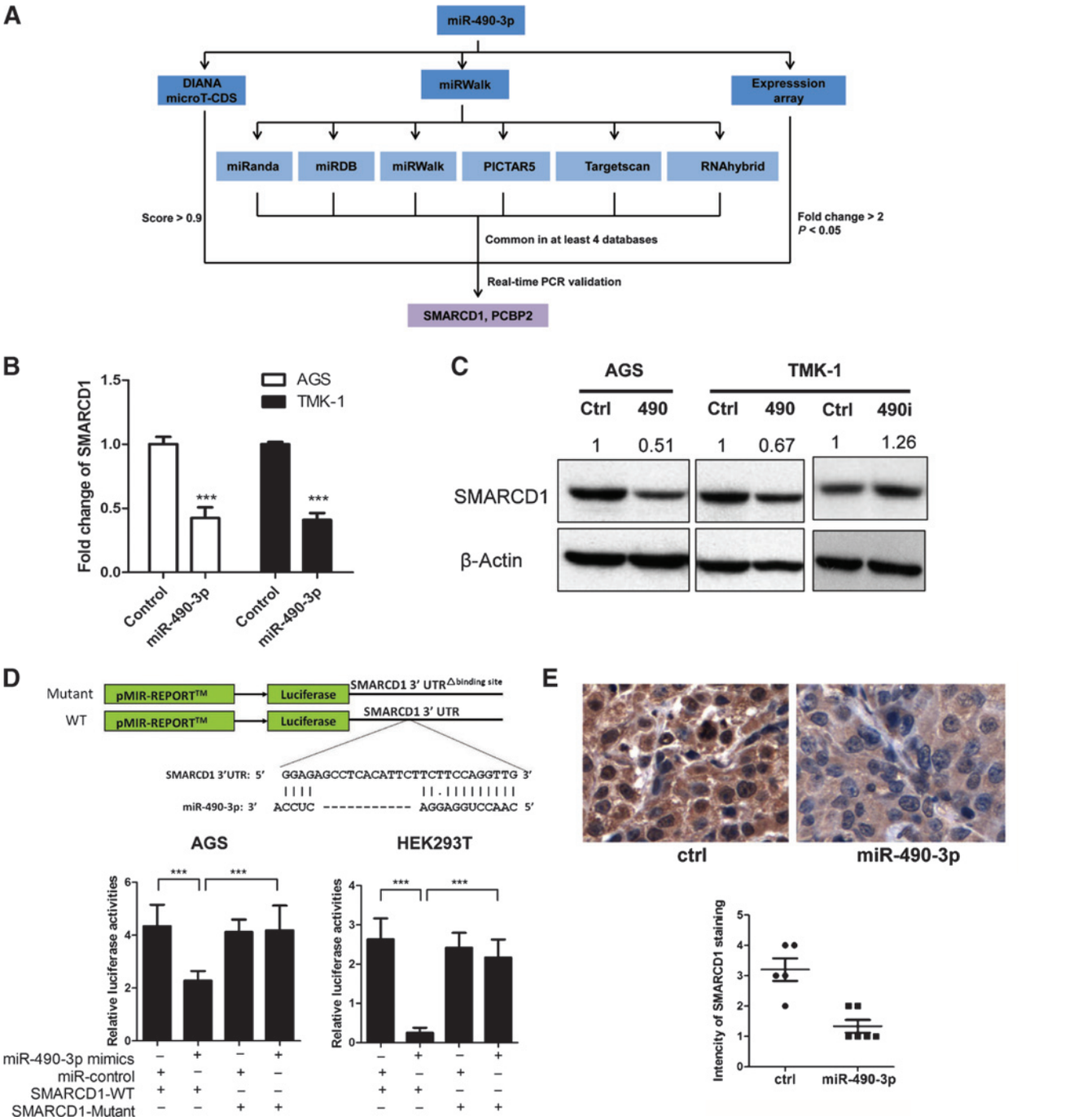


Figure 4

SMARCD1 as the direct target of miR-490-3p. A, an integrative approach combining computational predictions (DIANA and miRwalk) and expression array identified SMARCD1 as a putative target of miR-490-3p. B and C, downregulation of SMARCD1 mRNA (B) and protein (C) was confirmed in AGS and TMK-1 upon transfection with miR-490-3p mimics (490) by real-time PCR and Western blots, respectively. Restored expression of SMARCD1 was also observed upon transfection with miR-490-3p inhibitor (490i). D, luciferase reporter assay confirmed the direct physical interaction between miR-490-3p and SMARCD1 3′UTR in AGS and HEK293T cells. Mutation of putative miR-490-3p–binding site on SMARCD1 3′UTR abrogated the inhibitory effect of miR-490-3p on luciferase activity. E, protein expression of SMARCD1 was determined by IHC in TMK-1 gastric cancer xenografts; ***, $P < 0.001$ significantly different from the respective control group or between indicated groups.

Functional importance of SMARCD1 was corroborated by the fact that SMARCD1 knockdown phenocopied the effect of miR-490-3p and attenuated the oncogenic properties of miR-490-3p inhibitor. Overexpression of SMARCD1 also promoted oncogenic properties of gastric cancer cells. Importantly, we found that high SMARCD1 expression could predict poorer OS in

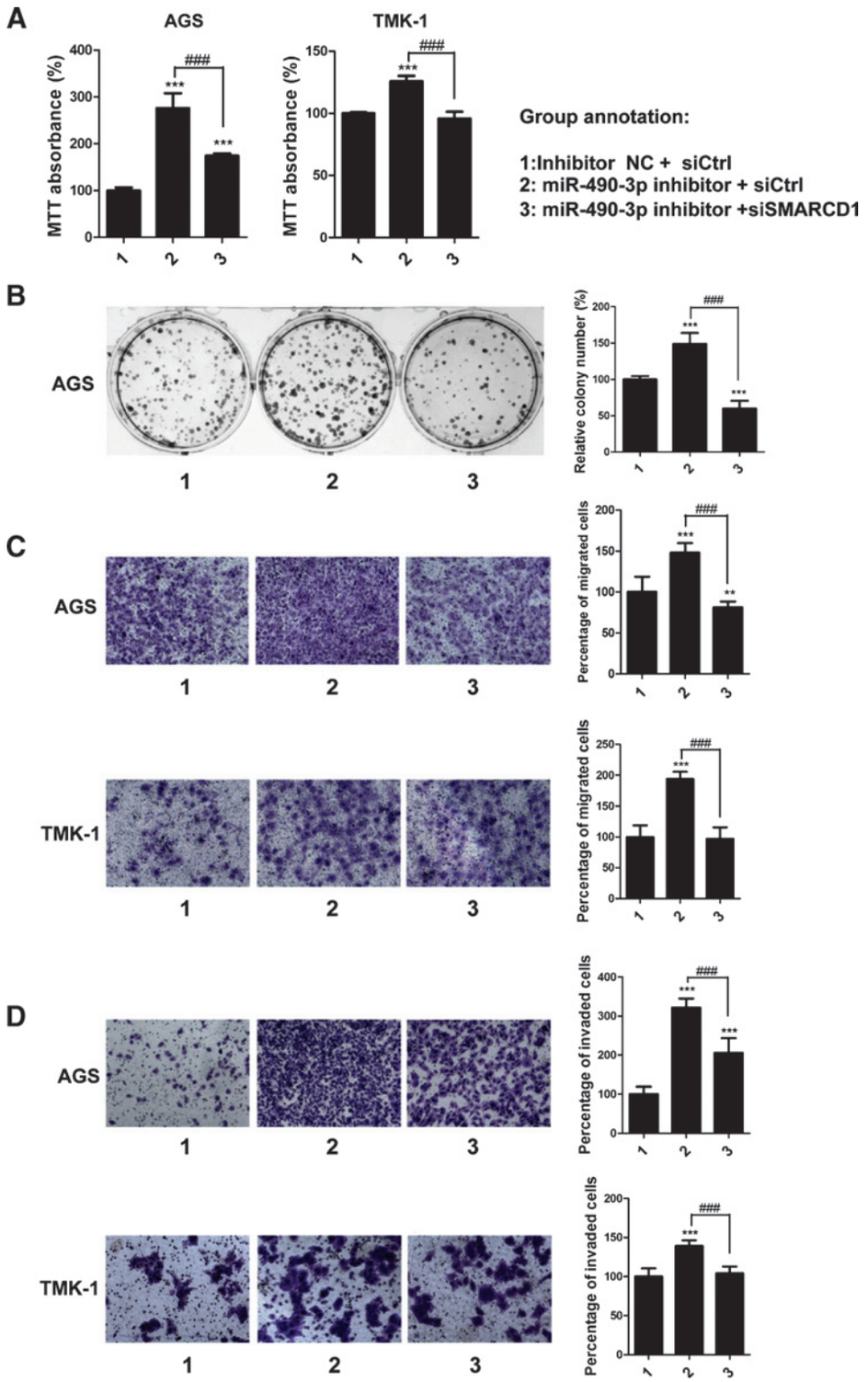


Figure 5
Attenuation of protumorigenic effects of miR-490-3p inhibitor by knockdown of SMARCD1. A–D, knockdown of SMARCD1 abrogated the stimulatory effect of miR-490-3p inhibitor on cell proliferation (96 hours posttransfection; A), colony formation (2 weeks posttransfection; B), migration (72 hours posttransfection; C), and invasion (96 hours posttransfection; D) of gastric cancer cells. SMARCD1 siRNA and miR-490-3p inhibitor were cotransfected into cells; ***, $P < 0.001$ significantly different between indicated groups; ###, $P < 0.001$ significantly different as compared with group 2.

patients with gastric cancer independent of TNM staging, suggesting that SMARCD1 might be used as a tissue prognostic marker. It is noteworthy that, although 5-aza-2′deoxycytidine could restore miR-490-3p expression in gastric cancer cell lines, the same chemical failed to repress SMARCD1 expression (data not shown). It could be explained by the possibility that DNA-demethylating agents could deregulate other targets that are upstream regulators of SMARCD1.

Deregulated transcriptional control by miRNAs has been a recurrent theme in gastric cancer (4). For instance, let-7 and

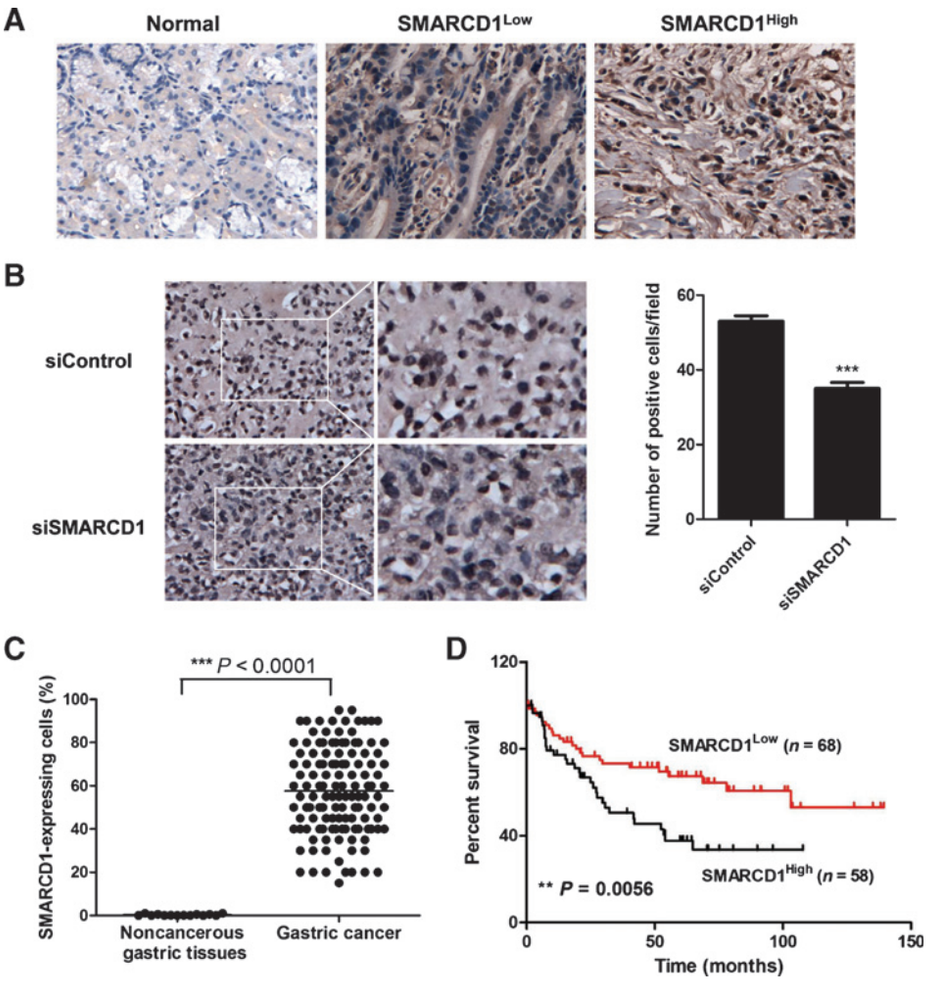


Figure 6
Overexpression of SMARCD1 in human gastric cancer tissues and its correlation with OS. A, representative immunohistochemical staining of SMARCD1 in normal (no immunoreactivity) and cancerous gastric cancer tissues (low and high SMARCD1 staining) are shown. B, nuclear immunoreactivity of SMARCD1 was significantly lower in AGS cells transfected with SMARCD1–siRNA. IHC was performed using AGS cell blocks. C, immunoreactivity of SMARCD1 was significantly higher in gastric cancer than that of normal gastric tissues. D, high SMARCD1 expression was correlated with shortened OS in patients with gastric cancer; ***, $P < 0.001$ significantly different from the respective control group.

Table 1. Univariate and multivariate Cox regression analysis

	Univariate analysis		Multivariate analysis	
Variables	**RR (95% CI)**	***P***	**RR (95% CI)**	***P***
Age, y	1.03 (1.01–1.06)	**0.013**	1.06 (1.03–1.09)	**<0.001**
Sex				
M	1.54 (0.83–2.83)	0.16	1.12(0.58–2.17)	0.731
F	1		1	
H. pylori infection				
Negative	1.43 (0.81–2.54)	0.218		
Positive	1			
Lauren type				
Intestinal	0.54 (0.22–1.35)	0.188		
Diffuse	1.33 (0.55–3.21)	0.532		
Mix	1			
Stage				
I	0.13 (0.06–0.30)	**<0.001**	0.13 (0.04–0.42)	**0.001**
II	0.17 (0.05–0.56)	**0.004**	0.15 (0.04–0.52)	**0.003**
III	0.38 (0.19–0.75)	**0.005**	0.27 (0.13–0.54)	**<0.001**
IV	1		1	
Lymph node metastasis				
No	0.24 (0.11–0.52)	**<0.001**	0.63 (0.19–2.06)	0.445
Yes	1		1	
SMARCD1				
Low	0.48 (0.27–0.82)	**0.008**	0.45 (0.25–0.81)	**0.008**
High	1		1	

NOTE: *P* values <0.05 are in bold.

miR-212, two commonly downregulated miRNAs in gastric cancer, could target HMGA2 and MeCP2, respectively (45, 46). The former is a nonhistone chromosomal protein that can promote the assembly of regulatory protein complexes at sites of transcription whereas the latter is a methyl-CpG–binding protein involved in epigenetic silencing of various tumor-suppressor genes (4). Increased expression of miR-27a in gastric cancer also represses prohibitin (47), a transcriptional coregulator of p53, E2F-1, and NF-κB (4). Here, we demonstrated that downregulation of SMARCD1, a reported chromatin-modifier and transcriptional coregulator of multiple transcription factors, could mediate the tumor-suppressing effects of miR-490-3p in gastric cancer. Our findings, together with reported recurrent mutations in *ARID1A* (15–17) also strongly suggest that aberrant chromatin remodeling consequent to genetic and epigenetic abnormalities in the SWI/SNF family is key to gastric tumorigenesis.

Taken together, induction of neoplastic changes in murine stomachs by *H. pylori* and a chemical carcinogen led to the discoveries of miR-490-3p downregulation and oncogenic function of its target SMARCD1 in human gastric cancer. SMARCD1, whose high-expression predicted poorer prognosis, functioned as an oncogene by promoting cell proliferation, migration, and

invasion of gastric cancer cells. It is expected that restoration of miR-490-3p function by directly delivering synthetic RNA into gastric tissues or targeting SMARCD1 may be viable strategies for the treatment of gastric cancer. With the recent entry of a miRNA-directed therapeutics into phase II clinical trials (48), it is propitious that our finding could be used for devising novel anticancer therapy in the future.

Disclosure of Potential Conflicts of Interest

F.K.L. Chan received speakers' bureau honoraria from and is a consultant/advisory board member for Eisai. No potential conflicts of interest were disclosed by the other authors.

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Grant Support

This work was supported by Health and Medical Research Fund 10090532 (Food & Health Bureau, Hong Kong), Research Fund for the Control of Infectious Diseases 08070172 (Food & Health Bureau, Hong Kong), and Shenzhen Basic Research Program (JC20110520111A).

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Received April 28, 2014; revised November 12, 2014; accepted November 22, 2014; published OnlineFirst December 12, 2014.

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