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Human Cancer Biology

Clinical Cancer Research

Genomic Loss of *miR-486* Regulates Tumor Progression and the *OLFM4* Antiapoptotic Factor in Gastric Cancer

Hue-Kian Oh[1], Angie Lay-Keng Tan[3], Kakoli Das[3], Chia-Huey Ooi[3], Nian-Tao Deng[3], Iain BeeHuat Tan[2], Emmanuel Beillard[3], Julian Lee[1], Kalpana Ramnarayanan[1], Sun-Young Rha[6], Nallasivam Palanisamy[4], P. Mathijs Voorhoeve[3], and Patrick Tan[1,3,4,5]

Abstract

Purpose: MicroRNAs (miRNA) play pivotal oncogenic and tumor-suppressor roles in several human cancers. We sought to discover novel tumor-suppressor miRNAs in gastric cancer (GC).

Experimental Design: Using Agilent miRNA microarrays, we compared miRNA expression profiles of 40 primary gastric tumors and 40 gastric normal tissues, identifying miRNAs significantly downregulated in gastric tumors.

Results: Among the top 80 miRNAs differentially expressed between gastric tumors and normals (false discovery rate < 0.01), we identified *hsa-miR-486* (*miR-486*) as a significantly downregulated miRNA in primary GCs and GC cell lines. Restoration of *miR-486* expression in GC cell lines (YCC3, SCH and AGS) caused suppression of several pro-oncogenic traits, whereas conversely inhibiting *miR-486* expression in YCC6 GC cells enhanced cellular proliferation. Array-CGH analysis of 106 primary GCs revealed genomic loss of the *miR-486* locus in approximately 25% to 30% of GCs, including two tumors with focal genomic losses specifically deleting *miR-486*, consistent with *miR-486* playing a tumor-suppressive role. Bioinformatic analysis identified the secreted antiapoptotic glycoprotein *OLFM4* as a potential *miR-486* target. Restoring *miR-486* expression in GC cells decreased endogenous *OLFM4* transcript and protein levels, and also inhibited expression of luciferase reporters containing an *OLFM4* 3′ untranslated region with predicted *miR-486* binding sites. Supporting the biological relevance of *OLFM4* as a *miR-486* target, proliferation in GC cells was also significantly reduced by *OLFM4* silencing.

Conclusions: *miR-486* may function as a novel tumor-suppressor miRNA in GC. Its antioncogenic activity may involve the direct targeting and inhibition of *OLFM4*. *Clin Cancer Res; 17(9); 2657–67. ©2011 AACR.*

Introduction

Gastric cancer (GC) is the second leading cause of global cancer mortality with a particularly high incidence in many Asian countries (1). Most GC patients are diagnosed with advanced stage disease and show extremely poor prognosis (2). The 5-year survival rate for patients with stage II disease ranges from 30% to 50%, falling to 10% to 25% for stage III patients (3, 4). Disease recurrence is also common, with less than 20% of late stage patients surviving beyond 5 years (3, 4). Despite a global decline in incidence, GC remains a disease of outstanding morbidity and mortality. Achieving a better understanding of recurrent molecular aberrations associated with GC carcinogenesis might identify new diagnostic and treatment strategies for this disease.

Genetic factors implicated in GC development include somatic mutations in classical tumor-suppressor genes and oncogenes (*p53*, *β-catenin*), gene amplifications and deletions (e.g., *c-Met* and *ERBB2*), and epigenetic inactivation of *CDKN2A* (p16), *CDH1* (E-cadherin), *hMLH1*, and *RUNX3* (5, 6). Besides protein coding genes, recent evidence has shown an important role for noncoding RNAs in human cancer and in particular microRNAs (miRNA; ref. 7). miRNAs are naturally occurring small RNA molecules of approximately 22 nucleotides (8) which base-pair with complementary sequences located in the 3′ untranslated regions (UTR) of target genes, causing either target mRNA degradation or reduced protein translation (9, 10). An individual miRNA can regulate a large number of target mRNAs (11), and conversely a single gene may be regulated

Authors' Affiliations: [1]Cellular and Molecular Research and [2]Division of Medical Oncology, National Cancer Centre of Singapore; [3]Cancer and Stem Cell Biology, Duke-NUS Graduate Medical School; [4]Genome Institute of Singapore; [5]Cancer Science Institute of Singapore, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; and [6]Department of Internal Medicine, Yonsei Cancer Center, Yonsei University College of Medicine, Seodaemun-Ku, Seoul, Korea

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

Current address for N. Palanisamy: Michigan Center for Translational Pathology and Departments of Pathology, Ann Arbor, MI 48109.

Corresponding Author: Patrick Tan, Cancer and Stem Cell Biology, Duke-NUS Graduate Medical School, 8 College Road, Singapore 169857. Phone: 65-65161783; Fax: 65-62212402. E-mail: gmstanp@duke-nus.edu.sg

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Translational Relevance

Gastric cancer (GC) is the second leading cause of global cancer mortality. In this study, we carried out microRNA (miRNA) profiling of primary GCs and cell lines to identify *hsa-miR-486*-5p (*miR-486*) as a significantly downregulated miRNA in GC. Subsequent functional characterization revealed that *miR-486* inactivation is required for the expression of several pro-oncogenic traits, and that this is likely mediated through *miR-486* targeting the *OLFM4* antiapoptotic factor. *miR-486* is located at Chr 8p11, a region of frequent genomic loss in multiple cancers. Consistent with *miR-486* playing a tumor-suppressor role, we observed by array-CGH frequent genomic deletion of *miR-486* in 20% to 30% of GCs. *miR-486* may thus represent a novel tumor-suppressor miRNAs in GC inactivated through genomic deletion. By understanding the mechanism and function of *miR-486* as a tumor suppressor, it may be possible to develop *miR-486* as a diagnostic or therapeutic agent in GC treatment.

by multiple independent miRNAs. The ability of miRNAs to affect multiple downstream target genes suggests that miRNAs may be particularly adept at broadly modulating the activity of multiple cellular pathways (9, 10). In cancer, miRNAs can exert either oncogenic or tumor-suppressive roles (12). Oncogenic miRNAs such as *miR-21*, *mir-155*, *mir-17–92*, and *miR-372/miR-373* are frequently upregulated in tumors and can negatively regulate tumor-suppressor genes such as *Bim*, *PTEN*, and *LATS2* (12–14). Conversely, tumor-suppressive miRNAs, which are usually underexpressed in tumors, have been shown to inhibit oncogenes such as *RAS*, *BCL2*, and *MYC* (12). Some examples of tumor-suppressor miRNAs include *let-7*, *mir-15*, and *mir-16*.

In GC, several miRNA profiling studies have been reported (15, 16). However, most of these previous studies have largely focused on characterizing oncogenic miRNAs (e.g., *miR-21*, *miR-27a*, and the *miR106b-25* cluster; refs. 17–19). In contrast, relatively few tumor-suppressor miRNAs in GC are known, and although candidates such as *miR-141*, *miR-143*, *miR-145*, *miR-9*, and *miR-218* have been proposed as possible tumor-suppressor miRNAs, their functions and target genes have not been extensively explored (20–24). Here, we compared miRNA expression profiles of gastric tumors and matched normal tissues to identify *hsa-miR-486-5p* (*miR-486*) as a significantly downregulated miRNA in GC. Subsequent functional characterization revealed that *miR-486* inactivation is required for the expression of several pro-oncogenic traits, which may involve *miR-486* targeting the *OLFM4* antiapoptotic factor. Importantly, we observed frequent and focal genomic deletions of the *miR-486* locus in 20% to 30% of GCs, consistent with *miR-486* playing a tumor-suppressor role. Finally, *miR-486* downregulation has also been observed in several other human cancers (25), it is thus possible that *miR-486* may exert important tumor-suppressive functions in other cancer types besides GC.

Materials and Methods

Primary gastric cancer samples and cell lines

Primary gastric tumors and adjacent matched normal gastric tissues were obtained from the National Cancer Centre Singapore and the Singhealth Tissue Repository. Primary samples were collected with signed patient informed consent and with approval from institutional review boards. GC cell lines AGS, Kato III, SNU1, NCI-N87, and Hs746T were obtained from the American Type Culture Collection (ATCC) and AZ-521, TMK1, MKN1, MKN7, MKN45 cells were obtained from the Japanese Collection of Research Bioresources (JCRB). SCH cells were a gift from Yoshiaki Ito (Cancer Sciences Institute of Singapore). YCC1, YCC3, YCC6, YCC7 cells were a gift from Sun-Young Rha (Yonsei Cancer Center, South Korea). All cell lines were tested and authenticated by the respective cell line bank (ATCC, JCRB) or the originating institution (YCC) by several methods including DNA fingerprinting and/or cytogenetics. Prior to the commencement of this study, we independently re-authenticated the cell lines by comparing their genome-wide copy number (array-CGH) and mutational profiles to the published literature.

miRNA expression profiling

Total RNA was extracted from primary tissues and cell lines by using the miRVana miRNA Isolation Kit (Ambion, Inc.) according to the manufacturer's instructions. RNA samples were hybridized to Agilent Human miRNA Microarrays (V2) representing 723 human and 76 human viral miRNAs, and scanned using an Agilent DNA Microarray Scanner (Model G2565BA). miRNA expression values were normalized against background signals by Feature Extraction Software (Agilent). The miRNA data were also subjected to a log 10 transformation followed by median centering across probes, prior to in-depth analysis. The miRNA expression data have been deposited into GEO under accession number GSE23739.

Microarray data analysis

Differentially expressed miRNAs were identified by using the significance analysis of microarrays (SAM) program in BRB-ArrayTools (http://linus.nci.nih.gov/~brb/tool.htm), using a false discovery rate (FDR) cutoff of less than 0.01. Significance of Pearson correlations (R) between 2 N-element vectors were estimated from the Student t distribution, against the null hypothesis that the observed value of $t = R/\sqrt{[(1 - R^2)/(N - 2)]}$ arises from a population in which the true correlation coefficient is zero. Of 146 differentially expressed miRNAs initially identified by SAM (FDR < 0.01), we focused on the top 40 miRNAs exhibiting the highest positive log fold change (most

upregulated) and the top 40 miRNAs with the lowest negative log fold change (most downregulated). Among the 40 most significant downregulated miRNAs in tumors, we triaged 16 candidate tumor-suppressor miRNAs meeting the additional criterion of detectible expression in an independent normal stomach sample from a healthy patient (Stratagene), at levels greater than the median expression in GC cell lines.

Real-time quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) was carried out by using an ABI7900HT Fast real-time PCR system (Applied Biosystems). TaqMan Universal PCR Master Mix (Applied Biosystems), TaqMan Reverse Transcription Kit, and TaqMan MicroRNA Assay kits (Applied Biosystems) were used to measure *miR-486* expression levels according to the manufacturer's instructions. Each PCR was normalized against an *RNU6B* internal control. All PCR reactions were done in triplicate.

Additional methods

Detailed methods on *miR-486* expression and silencing, *OLFM4* silencing and overexpression, cell proliferation and anchorage-independent growth assays, cell migration and invasion assays, array-CGH assays and copy number analysis, miRNA target prediction, Western blotting, *OLFM4* 3′-UTR Luciferase Reporter Assays, and *in situ* hybridization are reported in the Supplementary Data.

Results

Identification of differentially regulated microRNAs in primary gastric tumors

To identify miRNAs exhibiting expression changes in GC, we profiled 40 gastric tumors and 40 noncancerous gastric tissues on Agilent miRNA microarrays measuring approximately 800 miRNAs (723 human and 76 viral miRNAs). Gastric tumors are traditionally classified by histology into 2 major groups-–intestinal (expanding or differentiated) and diffuse (infiltrative or undifferentiated; ref. 26). Our analysis covered both GC subtypes, because half of the tumors were intestinal-type GC (20 samples), whereas the other half were diffuse-type cancers. An initial unsupervised analysis where the gastric samples were clustered by using miRNAs detectibly expressed in 25% or more of the samples (329 probes) resulted in an overall partitioning of gastric tumors away from normals, indicating the existence of pervasive miRNA expression differences between the 2 groups (Supplementary Fig. S1). We focused our analysis on miRNAs exhibiting striking differences in expression between gastric normals and tumors. Using SAM, we identified 80 miRNAs that were significantly differentially expressed between the 2 groups (FDR < 0.01; Fig. 1A; Supplementary Table S1 provides a list of the top 80 miRNAs).

We divided the differentially regulated miRNAs into 3 distinct classes on the basis of their global expression patterns. The first class of miRNAs exhibited high expression in tumors relative to normals—these may represent potential pro-oncogenic miRNAs contributing to GC development and progression. Supporting this idea, among the most highly expressed miRNAs in this class were *miR-21*, *miR-27a*, and *miR-17*—3 miRNAs that have been functionally confirmed to exert oncogenic functions

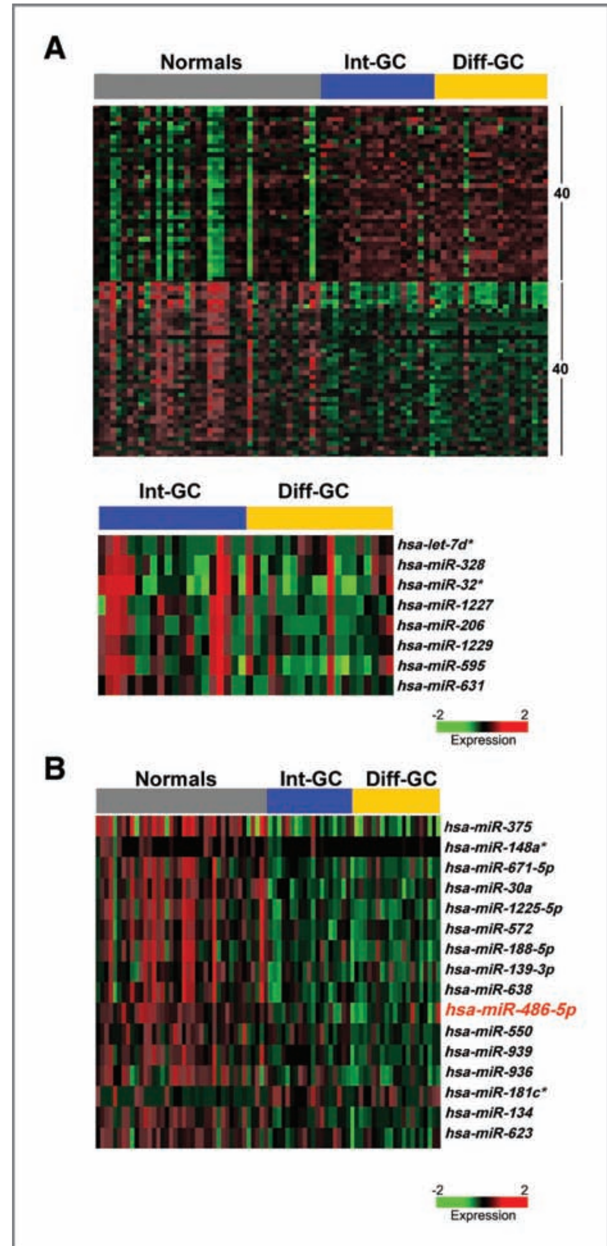

Figure 1. Global identification of differentially expressed miRNAs between gastric normal tissues and tumors. A, identification of differentially expressed miRNAs. Top, expression heat map showing expression of 80 differentially expressed miRNAs between and 40 normal gastric tissues (gray bar) and 40 GCs (blue and yellow bars; FDR < 0.01, using SAM, see Materials and Methods). Bottom, differential expression of miRNAs between intestinal-type and diffuse-type GCs (Int-GC and Diff-GC, respectively) analyzed from a separate SAM run (FDR < 0.01). B, heat map showing differential expression of 16 candidate tumor-suppressor miRNAs. Expression of *miR-486* is highlighted in red type.

in multiple cancer types including GC (17, 18). The reidentification of these known oncogenic miRNAs supports the biological validity of the miRNA microarray data. Interestingly, we also observed upregulation of *hsa-miR-16* and *hsa-miR-214* in GC—2 miRNAs that have been previously reported to be downregulated in prostate and cervical cancers (27, 28). It is thus possible that certain miRNAs may exert either pro or antioncogenic functions that are dependent on tissue type.

The second class comprised miRNAs exhibiting downregulation in tumors relative to normals—such miRNAs might represent candidates for potential tumor-suppressor miRNAs. Given the relative lack of validated tumor-suppressor miRNAs in GC compared with oncogenic miRNAs, we decided to focus on this list for further analysis. Using multiple filtering criteria (see Materials and Methods), we nominated a set of 16 candidate tumor-suppressor miRNAs (Fig. 1B). Providing confidence in our filtering criteria, among the 16 candidates we reidentified *mir-375*, a known tumor-suppressor miRNA in GC (29). These miRNAs are further considered in the next section.

We also detected a third and smaller class of miRNAs that were differentially expressed between intestinal and diffuse-type GCs (Fig. 1A, bottom). In general, these miRNAs (*hsa-let7d**, *hsa-miR-328*, *hsa-miR-32**, *hsa-miR-1227*, *hsa-miR-206*, *hsa-miR-1229*, *hsa-miR-595*, and *hsa-miR-631*) were largely downregulated in diffuse-type GCs compared with intestinal-type GCs. Although not the primary focus on this study, these results raise the possibility that differences in miRNA expression may also exist between the 2 major histologic subtypes of GC.

Expression patterns of *miR-486*, a candidate tumor-suppressor miRNA in GC

Hypothesizing that some of the 16 nominated miRNAs exhibiting decreased expression in GC might represent bona fide GC tumor suppressors, we selected a handful for functional validation. Here, we report our findings for the first of these candidates, *hsa-miR-486–5p*, hereafter referred to as *miR-486*. *miR-486* is located on chromosome 8p11 within intron 41 of the *Ankyrin-1* (*ANK1*) gene (Fig. 2A). *miR-486* is transcribed from an alternative promoter within intron 40

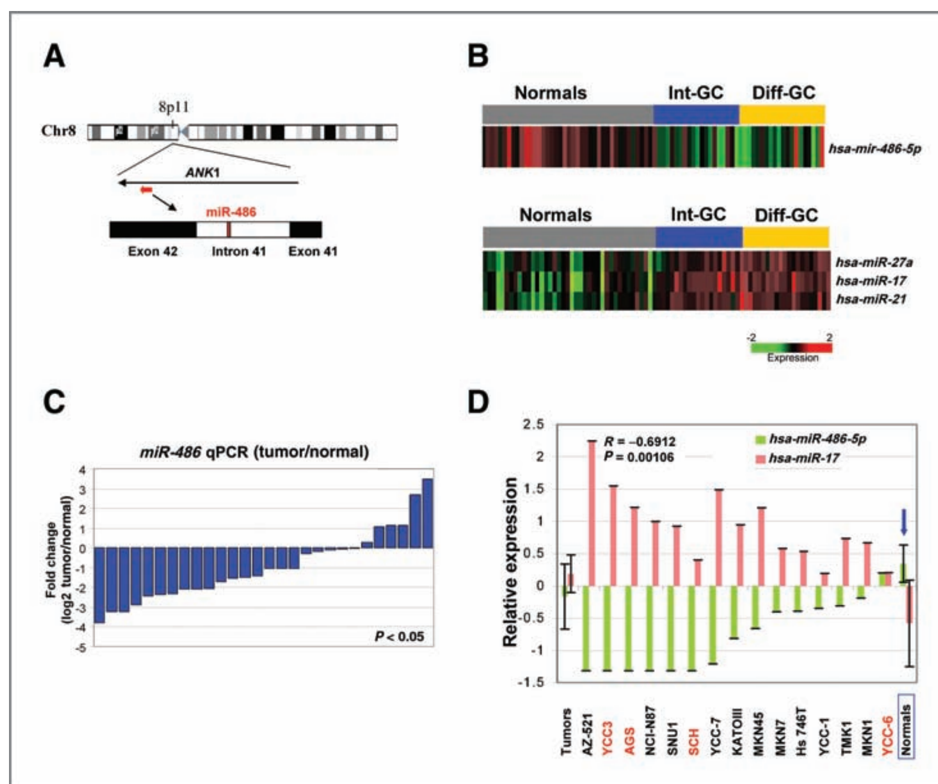

Figure 2. Expression of candidate tumor-suppressor *hsa-miR-486*-5p in gastric tumors and cell lines. A, genomic location of *miR-486*. *miR-486* is located on chromosome 8p11 within the *ANK1* gene, between exons 41 and 42. *miR-486* is transcribed from the same strand from an alternative promoter located in intron 40 of the *ANK1* gene. B, reciprocal expression of *miR-486* compared with 3 previously reported oncogenic miRNAs (*mir-17*, *mir-21*, and *mir-27a*) across gastric normal tissues and GCs. C, quantitative PCR (qPCR) analysis showing relative expression of *miR-486* in 29 primary GC tissues compared with matched adjacent normal tissues. Quantifications were measured by TaqMan real-time PCR. Each column represents an individual tumor/normal pair. Fold changes (tumor/normal) were transformed to log 2 values (*y*-axis). *P* values denotes the significance of fold change observed. D, expression of *miR-486* and mir-17 in primary gastric tissues (normals and tumors) and GC cell lines. The *x*-axis depicts primary GCs (first column, $n = 40$, median), 15 GC cell lines, and primary normal gastric tissues (blue arrow and box, last column, $n = 40$, median). Expression of *miR-486* is in green whereas *mir-17* is in pink (color legend). Error bars indicate respective standard deviations across tumors or normals. *R* denotes the Pearson correlation between the 2 miRNAs. *P* values denoting the significance of the correlation coefficient *R*. Cell lines selected for functional analysis are highlighted in red (YCC3, AGS, SCH, YCC6).

of the *ANK1* gene (30), and has been previously shown to regulate phosphoinositide 3-kinase signaling in muscle cells by targeting the *PTEN* gene (30). In contrast to its role in muscle development, the role of *miR-486* in epithelial tumorigenesis is currently unclear.

Three observations suggest that *miR-486* may play a potential tumor-suppressive role in GC. First, besides exhibiting reduced expression in tumors compared with the normal tissues, *miR-486* is expressed in a strikingly reciprocal pattern to the oncogenic miRNAs *miR-17*, *miR-21*, and *miR-27a* (Fig. 2B, bottom). Second, to validate the microarray results, we carried out qRT-PCR to directly measure *miR-486* expression levels in a cohort of 28 primary GC tissues and matched adjacent normal tissues. Eighty percent of the tumors expressed decreased levels of *miR-486* expression by at least 2-fold compared with matched normal tissues, confirming that *miR-486* is expressed at significantly lower levels in GCs ($P < 0.05$, paired *t* test; Fig. 2C). *In situ* hybridization experiments on fixed gastric tissue sections confirmed *miR-486* expression in normal gastric epithelial cells, but reduced or absent expression in gastric tumors (Supplementary Fig. S2). Third, to further validate the reduced expression of *miR-486* in GC cells, we investigated *miR-486* expression in a panel of 15 GC cell lines. Similar to the primary tumors, 14 of 15 GC cell lines underexpressed *miR-486* while simultaneously overexpressing the *mir-17* oncogenic miRNA ($P = 0.001$; Fig. 2D). Taken collectively, these results suggest that *miR-486* may play a tumor-suppressive role in GC.

miR-486 expression inhibits proliferation, anchorage-independent growth, migration, and invasion in GC cells

To investigate the functional significance of *miR-486* downregulation in GC, we selected 3 GC cell lines (YCC3, AGS, and SCH) for further studies as these 3 lines express low levels of *miR-486* (Fig. 2D). Synthetic *miR-486* precursor molecules were transfected into these cells lines to restore *miR-486* expression, and restoration of *miR-486* expression in these cells was confirmed by qRT-PCR (Fig. 3A). First, we compared the cell proliferation rates of control and *miR-486* transfected cells at various time points. In all 3 cell lines, the growth of *miR-486* transfected cells was significantly reduced compared with cells transfected with negative-control miRNAs (Fig. 3B). This result suggests that restoring *miR-486* expression is sufficient to inhibit cellular proliferation in GC.

Second, to examine the importance of *miR-486* in the tumorigenesis of GC cells, we carried out anchorage-independent growth assays. Using a blasticidin selection protocol (see Materials and Methods), we generated stable pools of SCH, YCC3, and AGS cells expressing *miR-486* or empty vector controls. SCH and YCC3 cells transfected with empty vector controls grew well in soft agar, forming distinct colonies (Fig. 3C). In contrast, SCH and YCC3 cells expressing *miR-486* exhibited a dramatic reduction in the number of soft agar colonies (Fig. 3C), showing transforming abilities less than 50% of the control cells. Similar data were obtained for AGS cells (Fig. 3C, right). These results suggest that *miR-486* can suppress the tumorigenecity of GC cells *in vitro*.

Third, to assess the effect of *miR-486* in GC migration and invasion, we tested AGS cells stably expressing *miR-486* or empty vectors. AGS cells expressing vector controls migrated robustly in Transwell assays (Fig. 3D, top), whereas AGS cells overexpressing *miR-486* exhibited a significant reduction in migration capacity ($P < 0.05$, approximately 2-fold). Similarly, in invasion assays, AGS cells overexpressing *miR-486* exhibited a 2-fold reduced capacity for invasion compared with controls ($P = 0.05$; Fig. 3D, bottom).

Fourth, of the 15 GC cell lines tested in this study, we identified 1 line (YCC6) expressing above-average levels of *miR-486* (Fig. 2D). To investigate the cellular effects of silencing rather than overexpressing *miR-486*, we inhibited endogenous *miR-486* expression in YCC6 cells by transfecting the cells with *miR-486* inhibitors (*anti–miR-486*). Efficient inhibition of *miR-486* expression was confirmed by qRT-PCR (Fig. 3E). *miR-486*–suppressed YCC6 cells exhibited a modest but significant enhancement of cell proliferation compared with control transfected cells ($P < 0.05$; Fig. 3E). Taken collectively, these results indicate that restoring *miR-486* expression is sufficient to suppress several pro-oncogenic traits *in vitro*, whereas conversely suppressing *miR-486* expression is sufficient to enhance such traits, consistent with *miR-486* playing a tumor-suppressive role in GC.

Frequent genomic loss of *miR-486* in GC

A preliminary transcriptomic analysis of GC cell lines treated with inhibitors of DNA methyltransferase or histone deactylation inhibitors failed to provide evidence of epigenetic silencing of *miR-486* in GC (data not shown). Besides epigenetic silencing (31), genomic loss is another mechanism by which tumor-suppressor miRNAs can be downregulated in cancer (32). Notably, *miR-486* is located on chromosome 8p11, a frequent region of loss of heterozygosity in many cancers, including GC (33, 34). Although *ANK1*, the gene within which *miR-486* lies, has been proposed as a potential tumor-suppressor gene in this region (34, 35), there is in reality very little functional evidence supporting an antioncogenic role for *ANK1*. These findings raise the possibility that other genetic elements on 8p11 lying close to *ANK1*, such as *miR-486*, may represent important driver elements for the frequent genomic losses associated with this region in cancer.

To investigate whether *miR-486* might be genomically deleted in GC, we analyzed an in-house array-CGH database of 106 gastric tumors profiled on Agilent 244K microarrays. Strikingly, we obtained genomic loss of the *miR-486* locus within a window of 500 kb on chromosome 8 in 25% to 30% of GCs (Fig. 4A). In individual GCs, the size of the genomic deletion ranged from 100 to 300 kb. Moreover, arguing against the possibility of another nearby gene driving these deletions, 2 tumors (GC990187 and GC200088) exhibited highly focal genomic regions specifically deleting

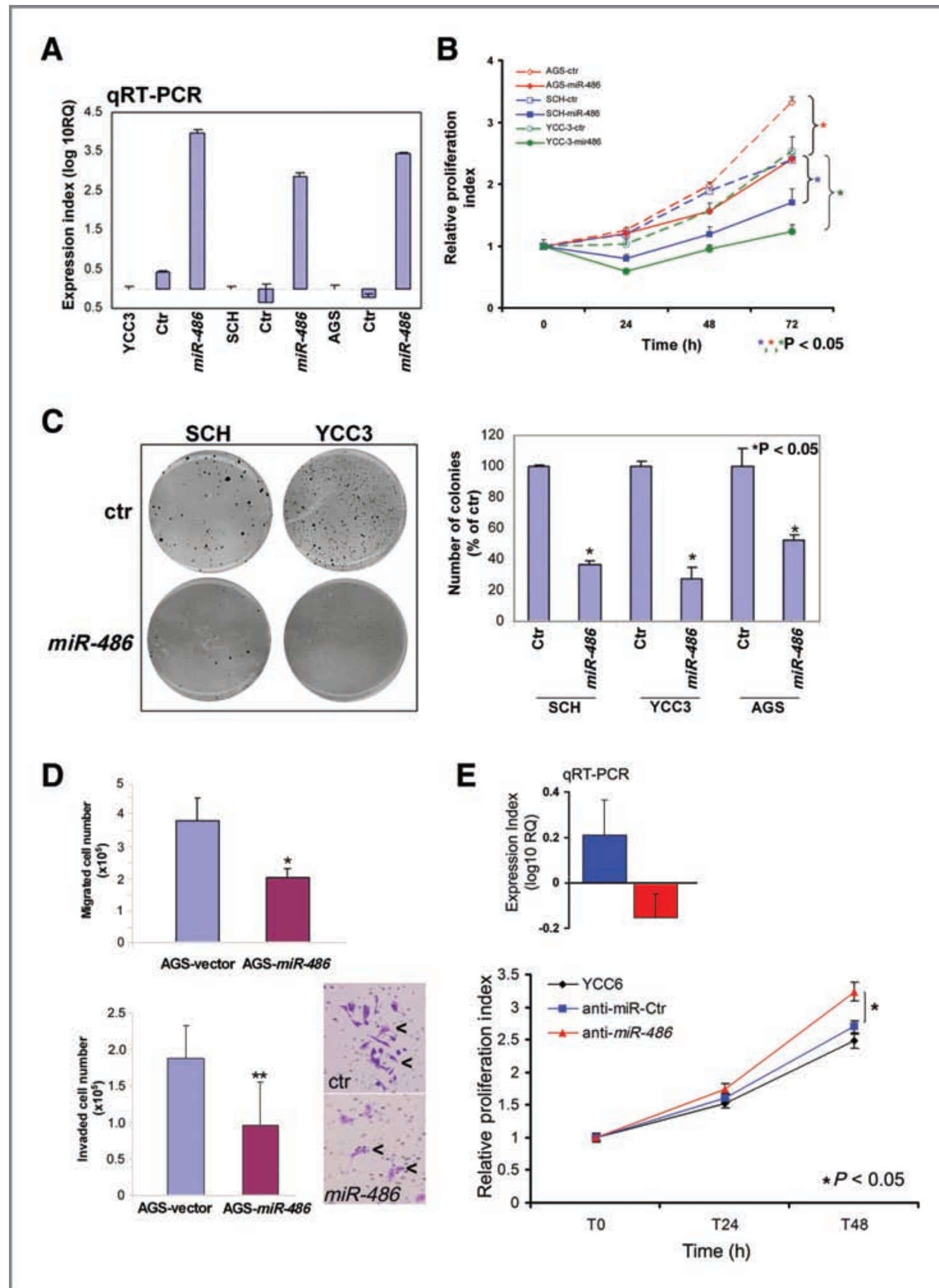

Figure 3. *miR-486* expression modulates multiple pro-oncogenic traits. A, restoration of *miR-486* expression in GC cell lines. YCC3, SCH, and AGS cells were transfected with control (Ctr) or *miR-486* precursors, qRT-PCR was carried out to assess relative *miR-486* expression. B, *miR-486* expression suppresses cellular proliferation in GC cell lines. *miR-486*–transfected cell lines were assessed for cell proliferation at 24, 48, and 72 hours posttransfection. Triplicate experiments were conducted for each set. *, $P < 0.05$, *t* test; points, mean; bars, SD. C, *miR-486* expression suppresses anchorage-independent cell growth. Left, soft-agar colony formation assay reduction showing reduced in colony numbers in SCH and YCC3 stably transfected with *miR-486* or empty vector control. Right, the quantification of colonies observed. Triplicate experiments were conducted for each cell lines. Columns, mean; bars, SD; *, $P < 0.05$, *t* test. D, *miR-486* reduces motility and invasiveness of AGS cells. Top, migration of AGS cells stably expressing *miR-486* or vector controls, measured by a Transwell migration assay. Columns, mean; bars, SD; *, $P < 0.05$. Bottom, invasion of AGS cells stably expressing *miR-486* or vector controls, measured by a Matrigel assay. Insert, phase contrast microscopy of the crystal-violet stained cells in Matrigel. Columns, mean; bars, SD; **, $P = 0.059$. E, inhibition of *miR-486* expression promotes cellular proliferation in YCC6. Top, *miR-486* inhibitor and negative control inhibitor [anti–*miR-486* (red) and anti–miR-ctr (blue)] transfected cells were assessed for *miR-486* expression by qRT-PCR. Bottom, cell proliferation levels were assayed at 24 and 48 hours posttransfection. Triplicate experiments were conducted for each set. Points, mean; bars, SD; significance for the difference in growth denoted by *, $P < 0.05$.

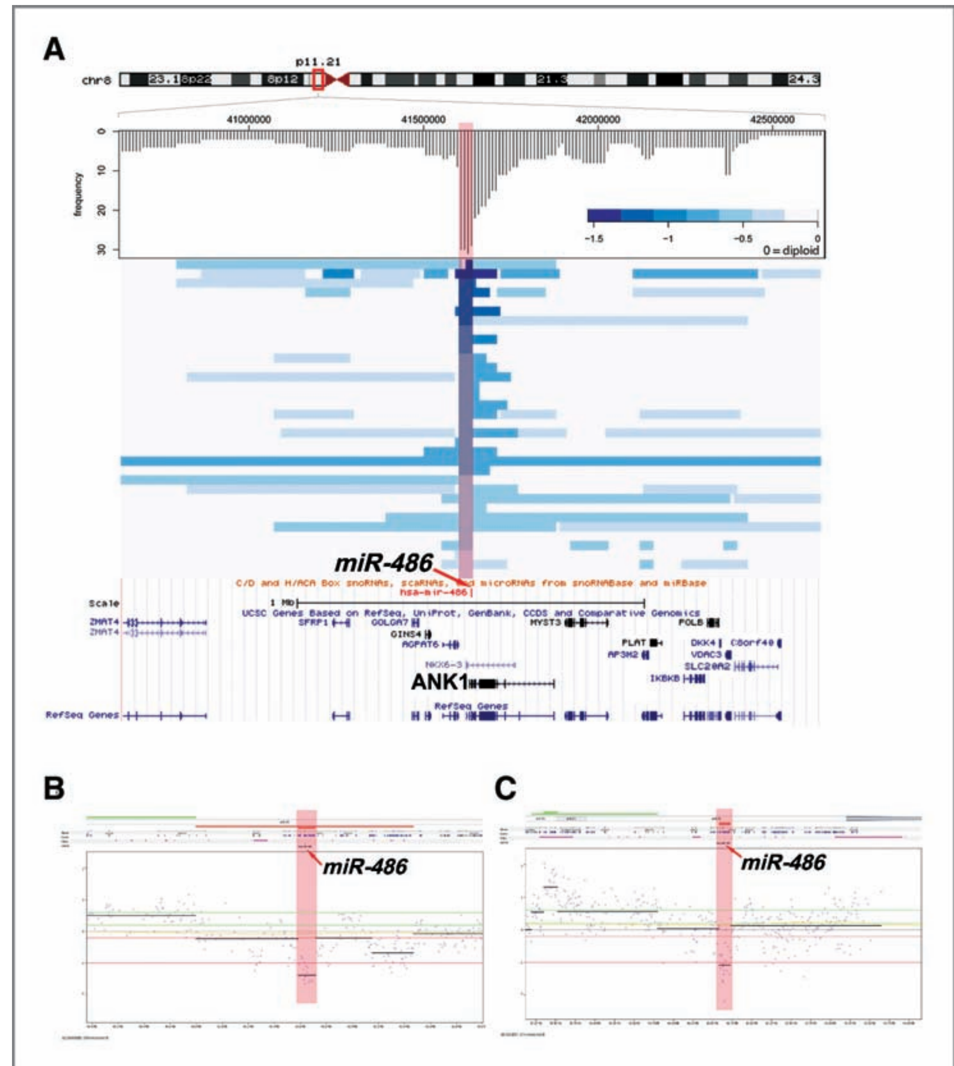

Figure 4. Genomic Loss of *miR-486* in primary GCs. A, recurrent genomic loss of *miR-486*. Red bars highlight the *miR-486* locus. Top, genomic location of *miR-486* on Chr 8p11.21. Middle, histogram showing frequency of genomic loss in this region across 106 primary GCs. Twenty-eight percent of GCs (30 tumors) are observed to exhibit loss of the miR-486 locus (blue horizontal bars below histogram). Genomic loss of the miR-486 locus in individual samples. Only samples with *miR-486* loss are shown. The color gradient depicts the extent of copy number deletion. Bottom, genome browser view of the Chr 8p11.21 region showing *miR-486* and adjacent genes such as *ANK1*. B and C, focal deletion of *miR-486* in 2 gastric tumor samples: 2000088 and 990187. The copy number log-ratio data are shown together with segments identified. The *miR-486* locus (with focal deletion) is highlighted in red.

the *miR-486* locus [GC990187—chr8:41,582,276-41,727,172 (135 kb); GC200088—chr8:41,582,276-41,717,096 (145 kb); Fig. 4B and C], whereas retaining a portion of the *ANK1* gene. We also observed a genomic deletion at the *miR-486* region in the GC cell line SCH (data not shown). These results indicate that the *miR-486* genomic locus is frequently deleted in GCs, supporting the notion that *miR-486* is a tumor-suppressor miRNA.

The antiapoptotic factor *OLFM4* is a direct *miR-486* target

To better understand the mechanisms underlying the tumor-suppressive capacities of *miR-486*, we searched for candidate *miR-486* target genes with potential pro-oncogenic functions. Using 2 miRNA target prediction programs (TargetScan 5.1 and miRanda v3.0), we identified 17 candidate *miR-486* target genes commonly predicted by both programs (Fig. 5A). Among these 17 genes, 2 (*TOB1* and *ARID1A*) have been previously shown to be associated with tumor-suppressive functions (36, 37). More importantly, we identified *SP5* and *OLFM4*, as potential pro-oncogenic *miR-486* target genes (38, 39). Our attention was specifically drawn to *OLFM4* (Olfactomedin-4/GW112), as *OLFM4* has been previously reported to be highly expressed in gastric tumors and specifically GC cells compared with normal tissues (39–41). Both prediction programs identified one potential *miR-486* binding site in the *OLFM4* 3′-UTR.

Functionally, *OLFM4* has also been shown to behave as an antiapoptotic factor and to promote tumor growth and invasion (see Discussion; refs. 42, 43). Confirming previous reports, we found that *OLFM4* was indeed highly expressed in GCs compared with gastric normals ($P < 0.001$; Fig. 5B). Also supporting recent findings, the high expression of *OLFM4* in tumors was largely associated with intestinal-type GCs (Table 1; ref. 40). No significant associations were observed between *OLFM4* expression with tumor stage, grade, or patient survival (data not shown).

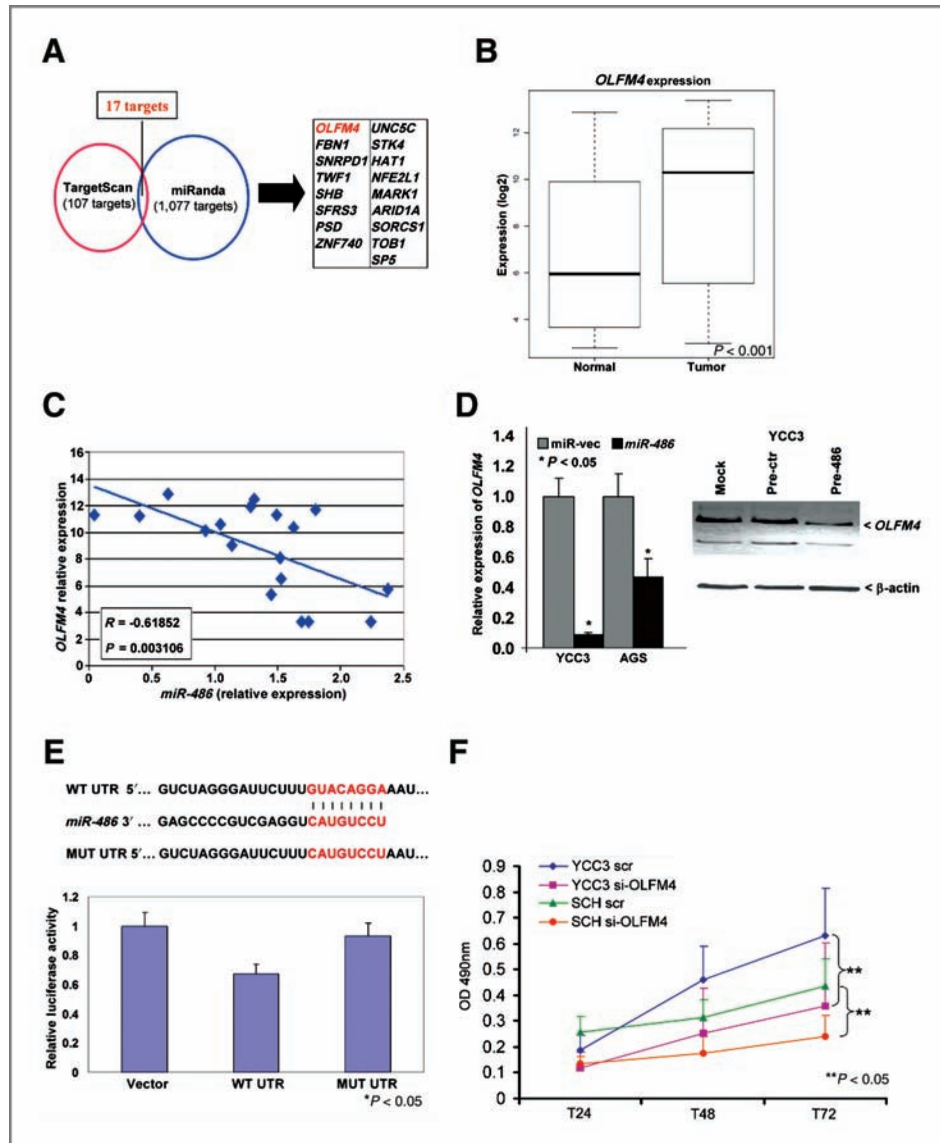

Figure 5. Direct regulation of Olfactomedin-4 (OLFM4) by *miR-486*. A, *miR-486* target prediction by miRanda v3.0 and TargetScan 5.1 algorithms. Seventeen targets were common to both prediction programs (genes in box). *OLFM4* is highlighted in red. B, *OLFM4* is highly expressed in primary gastric tumors compared with matched normal tissues ($P < 0.001$). C, OLFM4 and *miR-486* in intestinal-type primary GCs and matched normals. We observed a significant negative correlation of *miR-486* to *OLFM4* expression ($R = -0.61852$; $P = 0.003106$) in 11 intestinal-type GCs and 7 matched normal tissues analyzed. D, *OLFM4* protein levels are regulated by *miR-486*. Western blot analyses of *OLFM4* protein in cells transfected with *miR-486* or negative control mimics. *OLFM4* protein levels ware lower in YCC3 cells expressing *miR-486* as compared with control-miR–expressing cells. Similar reductions in *OLFM4* protein were also observed in *miR-486*–expressing AGS cells. Quantitative real-time PCR showed that *OLFM4* transcripts are also reduced in cells transfected with *miR-486* as compared with controls. (*, *t* test for *OLFM4* expression in *miR-486* vs. control cells). E, *OLFM4* is a direct target of *miR-486*. The predicted *miR-486* target region found in the *OLFM4* mRNA 3′-UTR was cloned downstream of luciferase in a pMIR-Report-luciferase reporter vector. Reporter constructs were cotransfected with *miR-486* and negative control mimic molecules into AGS cells. Luciferase reporter assays were normalized to β-galactosidase activities and experiments were conducted in triplicates. Data were plotted after normalized against the negative control miRNA mimics. Columns, mean; bars, SD. F, silencing of *OLFM4* in GC cells YCC3 and SCH by siRNA reduces cell proliferation capacity. YCC3 and SCH cells were transfected with siRNAs against *OLFM4* or scrambled controls (scr, negative control). Experiments were conducted in triplicates. *y*-Axis denotes the absorbance at 490 nm (cell proliferation) and *x*-axis is the assay time-points. Points, mean; bars, SD; significance for the difference in growth is denoted by **.

To investigate relationships between endogenous *miR-486* and *OLFM4* expression, we analyzed 11 intestinal-type primary GCs and matched nonmalignant tissues for which both *miR-486* and *OLFM4* expression were available. *miR-486* was significantly negatively correlated to *OLFM4* expression ($R = -0.619$; $P = 0.0031$; Fig. 5C), consistent with *miR-486* targeting *OLFM4 in vivo*. To directly assess the functional impact of *miR-486* on *OLFM4* expression, we transfected GC cells (YCC3 and AGS) with *miR-486* precursors and measured endogenous *OLFM4* expression

Table 1. High *OLFM4* expression is associated with intestinal-type GC

Lauren's classification	Intestinal	Diffuse	Mixed	Total
OLFM4 low expression	37	42	8	87
OLFM4 high expression	52	24	11	87
Total	89	66	19	174

NOTE: A Pearson χ^2 statistic was used to test the association of the expression of *OLFM4* with GC histologic subtype (174 tumors). High *OLFM4* expression was positively associated with intestinal-type GC (Pearson χ^2 test, $P = 0.019$).

levels. Restoration of *miR-486* in YCC3 and AGS cells caused a decrease in *OLFM4* protein and transcript levels, relative to control-transfected cells (Fig. 5D). These results indicate that restoration of *miR-486* in GC cells is sufficient to suppress *OLFM4* expression.

We then carried out luciferase reporter assays to show a direct functional role of the predicted *miR-486* binding sites in the *OLFM4* 3′-UTR. Luciferase reporters were constructed containing either a wild-type *OLFM4* 3′-UTR sequence containing the *miR-486* binding site (WT-UTR), or a mutated *OLFM4* 3′-UTR where the *miR-486* seed sequence binding sites were altered (MUT-UTR; Fig. 5E). The WT-UTR and MUT-UTR luciferase reporter constructs were transfected into GC cells, along with *miR-486* or negative control miRNAs. Luciferase expression of the WT-UTR reporter was significantly decreased compared with MUT-UTR ($P < 0.05$) or vector-expressing cells in a *miR-486*–dependent manner, indicating that *miR-486* is able to reduce the reporter activity of WT-UTR but not MUT-UTR (Fig. 5E). This result strongly indicates that that *miR-486* directly targets the *OLFM4* 3′-UTR, resulting in the translation inhibition of *OLFM4* protein.

OLFM4 expression promotes GC cell proliferation and inhibits the antioncogenic effects of _miR-486_

OLFM4 has been proposed to promote tumor growth by functioning as an antiapoptotic protein (42, 43) attenuating the apoptotic function of GRIM-19, a cell-death regulatory protein. To establish a functional role for *OLFM4* in GC, we silenced *OLFM4* in YCC3 and SCH cells, and conducted cell proliferation assays. *OLFM4*-silenced YCC3 and SCH cells exhibited significantly slower cellular proliferation compared with control siRNA-treated cells (Fig. 5F). This result suggests that *OLFM4* activity may be required for GC development and progression. Interestingly, downregulation of *OLFM4* by *miR-486* also resulted in upregulation of GRIM-19, the proposed target of *OLFM4* (Supplementary Fig. S3).

Finally, to establish whether the antiproliferative effects caused by *miR-486* restoration might depend, at least in part, on suppression of *OLFM4* activity, we carried out rescue experiments in which YCC3 cells stably expressing *miR-486* (Y4 cells) were transfected with *OLFM4* expressing constructs. We found that Y4 cells overexpressing OLFM4 exhibited a significant increase in cell proliferation compared with control Y4 cells, comparable with parental YCC3 cells (Supplementary Fig. S4). These results suggest that the tumor-suppressive effects of *miR-486* are likely to be mediated, at least in part, through its effect on *OLFM4* activity. In summary, our data suggest that *miR-486* is a tumor suppressor in GC progression and its downregulation in GC by genomic deletion may facilitate tumor growth, in part, by causing *OLFM4* upregulation.

Discussion

Recent evidence has convincingly shown an important role for miRNAs in many human cancers (7, 12). In GC, previous miRNA profiling studies have led to the collective identification of approximately 80 miRNAs exhibiting dysregulated expression between tumors and normals. However, beyond their observed expression patterns, relatively few tumor-suppressor miRNAs have been functionally explored in GC, notable exceptions being *miR-375* (regulating *PDK1* and *14-3-3ζ*) and *miR-141*, whose downstream targets are still unclear (21, 29). Thus, the true biological relevance of many miRNAs in GC requires further investigation. Here, we identified *miR-486* as a candidate GC tumor suppressor. Restoration of *miR-486* in multiple GC cell lines significantly reduced several pro-oncogenic traits, including cell proliferation, anchorage-independent growth, and cell migration/invasion, whereas silencing of *miR-486* in YCC6 cells enhanced proliferation. To our knowledge, our study is the first to functionally explore the role of *miR-486* in cancer development and progression. However, it is worth noting that Navon and colleagues have recently reported that *miR-486* is under-expressed in several other cancer types besides GC (25). Thus, the tumor-suppressive role of *miR-486* in cancer may not be limited to GC alone and our findings may have relevance to other cancer types.

The exact mechanisms causing *miR-486* downregulation in cancers deserve further study. In this study, we found that approximately 25% to 30% of gastric tumors exhibited a genomic loss of the chromosome 8p11 region where *miR-486* is located. This frequency of 8p loss is comparable with previous array-based CGH studies of GC (44, 45). Moreover, genomic deletions in miRNAs have been reported as a mechanism for miRNA downregulation, as shown for *miR-101*, *miR-15a*, and *miR-16-1* (32, 46). However, because 70% of GCs did not exhibit observable genomic loss of *miR-486*, genomic deletions alone are unlikely to fully explain the pervasive downregulation of *miR-486* in GC. It is thus almost certain that other GC tumors must employ alternative mechanisms to achieve *miR-486* downregulation, such as epigenetic silencing or transcriptional repression, as reported for *miR-124* (47) or transcriptional suppression of the *miR-29a* promoter by *myc* (48). In our study, we did

not observe evidence of *miR-486* epigenetic regulation when GC cell lines were treated with either inhibitors of DNA methyltransferase or histone acetyltransferases; however, these experiments were far from exhaustive. More work is thus required to fully delineate the spectrum of mechanisms responsible for *miR-486* downregulation in GC.

Our study suggests that *OLFM4* is likely a direct target gene of *miR-486*. However, it is likely oversimplistic to expect that the antioncogenic effects of *miR-486* can be entirely explained by its ability to regulate a single gene alone, particularly because previous studies investigating the cellular functions of miRNAs have shown that a single miRNA can often regulate many genes and gene targets (11). Besides *OLFM4*, our bioinformatic analysis we identified 16 other potential *miR-486* target genes, several of which may function in cancer (e.g., *FBN1*, *HAT1*, *SP5*, *TOB1*, *ARID1A*, and *OLFM4*), and *miR-486* has also been shown to target *PTEN* in muscle cells (30). Nevertheless, *OLFM4* is likely to be a biologically relevant *miR-486* target in the context of GC. *OLFM4* has been reported to be overexpressed in various cancers including GC but also colon, breast, and lung cancers (41), and has been proposed as a potential serum biomarker of GC (39). Functionally, *OLFM4* has been shown to interact with GRIM19 (a cell-death regulatory protein), cadherins, and lectins, and *OLFM4* has been shown to inhibit apoptosis and promote tumor growth and invasion (41–43). However, a recent finding showed that *OLFM4* has a proapoptotic effect in myeloid leukemia cells (49). In our study, the biological relevance of *OLFM4* as a *miR-486* target was supported by showing that *OLFM4* silencing can reduce GC cellular proliferation, and that OLFM4 overexpression can rescue the antioncogenic effects of *miR-486*. Interestingly, despite *miR-486* being downregulated in both intestinal and diffuse-type GCs, we found that *OLFM4* overexpression was largely confined to intestinal-type GCs. It is possible that in diffuse-type GCs *OLFM4* might be targeted by additional miRNAs and not simply *miR-486*. Consistent with this notion, preliminary bioinformatic analysis suggests that the *OLFM4* gene may be targeted by more than 400 different miRNAs (data not shown). Thus, other miRNAs may also act to target *OLFM4* in diffuse-type GCs, whereas in intestinal-type GCs, *miR-486* regulation of *OLFM4* may exert a predominant role. In this regard, it is intriguing to note that *OLFM4* has also been recently reported to be a robust marker of intestinal stem cells (50).

In conclusion, our data suggest that *miR-486* may act as a novel tumor-suppressor miRNA in GC, and that its downregulation in gastric tumors may be required for GC development and progression. Besides providing insights in basic aspects of GC biology, our findings may also have translational relevance as miRNAs have also been proposed to represent potential therapeutic candidates. By understanding the mechanism and function of *miR-486* as a tumor suppressor, it may be eventually possible to develop *miR-486* as a therapeutic agent in GC treatment.

Disclosure of Potential Conflicts of Interest

A patent application describing this work has been filed on behalf of the authors by the Agency for Science, Technology and Research.

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