E2F1-Regulated MicroRNAs Impair TGFβ-Dependent Cell-Cycle Arrest and Apoptosis in Gastric Cancer

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SUMMARY

Deregulation of E2F1 activity and resistance to TGFβ are hallmarks of gastric cancer. MicroRNAs (miRNAs) are small noncoding RNAs frequently misregulated in human malignancies. Here we provide evidence that the *miR-106b-25* cluster, upregulated in a subset of human gastric tumors, is activated by E2F1 in parallel with its host gene, *Mcm7*. In turn, *miR-106b* and *miR-93* regulate E2F1 expression, establishing a miRNA-directed negative feedback loop. Furthermore, upregulation of these miRNAs impairs the TGFβ tumor suppressor pathway, interfering with the expression of CDKN1A (*p21*$^{Waf1/Cip1}$) and BCL2L11 (*Bim*). Together, these results suggest that the *miR-106b-25* cluster is involved in E2F1 posttranscriptional regulation and may play a key role in the development of TGFβ resistance in gastric cancer.

SIGNIFICANCE

MicroRNAs (miRNAs) are small noncoding RNAs that may regulate the expression of approximately 30% of all human genes, either inhibiting target mRNA translation or inducing its degradation. These genes are abnormally expressed in human malignancies, making their biological importance increasingly apparent. Gastric cancer causes 12% of all cancer-related deaths each year, a fact that calls for better treatments based on a deeper understanding of the molecular mechanisms underlying the onset of this disease. Here, we show that overexpression of the *miR-106b-25* cluster leads to deregulation of important cancer-related genes, such as the TGFβ effectors *p21*$^{Waf1/Cip1}$ and *Bim*, disrupting the G1/S checkpoint and conferring resistance to TGFβ-dependent apoptosis.

INTRODUCTION

Although the incidence of gastric cancer declined in Western countries from the 1940s to the 1980s, it remains a major public health problem throughout the world, being the second most widely diagnosed malignancy worldwide and cause of 12% of all cancer-related deaths each year (Uemura et al., 2001). Over 95% of gastric tumors are adenocarcinomas histologically classified either as intestinal or diffuse type (Lauren, 1965). The evolution of intestinal tumors has been characterized as progressing through a number of sequential steps. Among the others, two events are characteristic of gastric tumorigenesis: upregulation of E2F1 (Suzuki et al., 1999) and development of TGFβ resistance (Ju et al., 2003; Park et al., 1994).

E2F1 is a master regulator of cell cycle that promotes the G1/S transition transactivating a variety of genes involved in chromosomal DNA replication, including its own promoter (DeGregori, 2002). While overexpression of E2F1 is an oncogenic event per se that predisposes cells to transformation (Pierce et al., 1999), it also represents a potent apoptotic signal when occurring over a critical threshold (Lazzerini Denchi and Helin, 2005).

On the other hand, Transforming Growth Factor-β (TGFβ) is a cytokine playing a major role within the so-called morphogenetic program, a complex system of crosstalk between the epithelial and the stromal compartments that guides gastrointestinal cells toward proliferation, differentiation, or apoptosis (van den Brink and Offerhaus, 2007).

In this study, we explored the possibility that microRNAs (miRNAs) may be involved in gastric tumorigenesis. miRNAs are non-protein-coding genes thought to regulate the expression of up to 30% of human genes, either inhibiting mRNA translation or inducing its degradation (Lewis et al., 2005). Besides a crucial role in cellular differentiation and organism development (Kloosterman and Plasterk, 2006), miRNAs are frequently misregulated in human cancer (Lu et al., 2005; Volinia et al., 2006), and they can act as either potent oncogenes or tumor suppressor genes (Esquela-Kerscher and Slack, 2006).

Here we show that E2F1 regulates *miR-106b*, *miR-93*, and *miR-25*, a cluster of intronic miRNAs hosted in the *Mcm7* gene, inducing their accumulation in gastric primary tumors. Conversely, *miR-106b* and *miR-93* control E2F1 expression, establishing a negative feedback loop that may be important in preventing E2F1 self-activation and, possibly, apoptosis.

On the other hand, we found that *miR-106b*, *miR-93*, and *miR-25* overexpression causes a decreased response of gastric cancer cells to TGFβ interfering with the synthesis of *p21* and *Bim*, the two most downstream effectors of TGFβ-dependent cell-cycle arrest and apoptosis, respectively. Therefore, these miRNAs contribute to the onset of TGFβ resistance in cancer cells and may represent novel therapeutic targets for the treatment of gastric cancer.

RESULTS

Deregulation of miRNA Expression in Human Gastric Cancer

It is well documented that most gastric adenocarcinomas arise in the context of a chronic inflammatory background, frequently associated with *Helicobacter pylori* (HP) infection (Uemura et al., 2001). Nevertheless, the molecular mechanisms responsible for HP oncogenicity are poorly understood, although Th1 immune response seems to be critical in the development of preneoplastic lesions such as gastric atrophy and intestinal metaplasia (Houghton et al., 2002; Fox et al., 2000).

In the search of miRNAs potentially involved in gastric tumorigenesis, we analyzed global miRNA expression in 20 gastric primary tumors of the intestinal type, each one paired with adjacent nontumor gastric tissue from the same patient, and six gastric cancer cell lines using a custom miRNA microarray. To identify specific alterations associated with inflammation and/or preneoplastic lesions, we first compared nontumor tissues with histological signs of chronic gastritis (n = 13) versus otherwise normal mucosa (n = 7). Seven miRNAs were associated with chronic inflammation by unpaired significance analysis of microarrays (SAM), including *miR-155*, which is known to predispose to cancer (Costinean et al., 2006) and to play a major role in the regulation of immune response (Rodriguez et al., 2007; Thai et al., 2007) (Figure 1A; Table S1 available online).

We then examined the miRNA expression profile of gastric primary tumors and cancer cell lines: a total of 14 miRNAs exhibited a 2-fold or greater median overexpression in primary tumors compared to nontumor controls by paired SAM (Figure 1B; Table S2). Of these, 13 out of 14 ranked above the 80th percentile in all gastric cancer cell lines in terms of expression, except for miR-223, which was not expressed (Table S3). Only five miRNAs were downregulated in cancer (Figure 1B; Table S2). Microarray data were confirmed by stem-loop qRT-PCR for nine out of ten tested miRNAs (Table S4). Among the misregulated miRNAs, *miR-21*, *miR-223*, *miR-25*, and *miR-17-5p* showed the highest overexpression in tumors, with 4.5, 4.2, 3.7, and 3.7 median fold changes, respectively.

These results indicate that specific modifications in the miRNA expression pattern are characteristic of human gastric cancer since the earliest steps of tumorigenesis and involve miRNAs with known oncogenic properties, such as *miR-21* (Meng et al., 2006) and *miR-17-5p* (He et al., 2005).

miR-106b-25 Cluster Is Overexpressed in Gastric Cancer

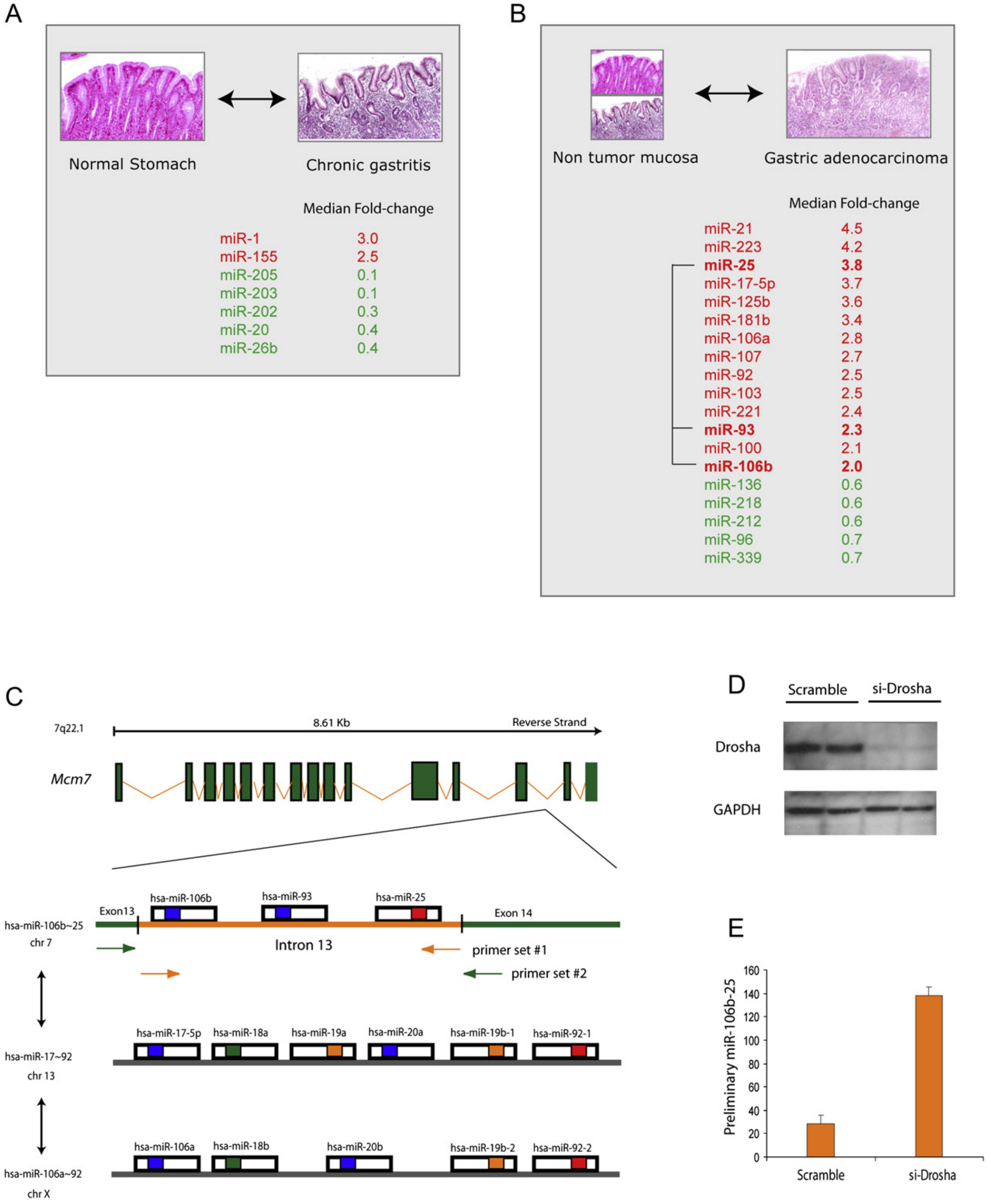
Among the overexpressed miRNAs, *miR-25* stood out as an attractive candidate for playing a role in gastric tumorigenesis. In fact, this was the third-most strongly upregulated miRNA in primary gastric tumors (median fold change: 3.7; range 1.0–26.8) and ranked among the most highly expressed miRNAs in all human gastric cancer cell lines (above 97th percentile). *miR-106b* (median fold change: 2.0; range 1.0–6.5) and *miR-93* (median fold change: 2.3; range 1.0–7.7) were also upregulated in primary tumors and highly expressed in all gastric cancer cell lines (above 82nd and 89th percentile, respectively).

These three miRNAs (hereafter *miR-106b-25*) are clustered in the intron 13 of *Mcm7* on chromosome 7q22 and actively cotranscribed in the context of *Mcm7* primary RNA transcript (Kim and Kim, 2007; Figures 1C–1E). Several studies reported the amplification of this region in gastric tumors (Weiss et al., 2004; Peng et al., 2003; Takada et al., 2005). However, we could not detect any amplifications of the *miR-106b-25* locus in our samples (data not shown), implying that other mechanisms must contribute to *miR-106b-25* overexpression in gastric cancer.

Mcm7 plays a pivotal role in the G1/S phase transition, orchestrating the correct assembly of replication forks on chromosomal DNA and ensuring that all the genome is replicated once and not more than once at each cell cycle (Blow and Hodgson, 2002). As overexpression of *Mcm7* has been associated with bad prognosis in prostate and endometrial cancer (Ren et al., 2006; Li et al., 2005), we hypothesized that *Mcm7* oncogenicity may be linked, at least in part, to overexpression of the hosted miRNAs. Moreover, the *miR-106b-25* cluster shares a high degree of homology with the *miR-17-92* cluster (Figure 1C), which appears to have an oncogenic role (He et al., 2005; O'Donnell et al., 2005; Dews et al., 2006).

These observations led us to pursue the *miR-106b-25* cluster as an interesting target for further studies. Given the possibility of cross-hybridization between homolog miRNAs, we first determined the specificity of stem-loop qRT-PCR. Primers for *miR-106b*, *miR-93*, and *miR-25* were highly specific, while *miR-17-5p* and *miR-92* probes cross-hybridized with *miR-106a* and *miR-25*, respectively (Figure S1A). Next, we used stem-loop qRT-PCR to assay the expression of mature miRNA species in an independent set of ten gastric primary tumors paired with nontumor gastric mucosa from the same patient. Mature *miR-106b*, *miR-93*, and *miR-25* were overexpressed in 6/10, 6/10, and 5/10 of these tumors, respectively, although there was not reciprocal correlation in their level of expression (Figure S1B).

To shed more light on this aspect, we examined miRNA precursor levels in the same tumors by conventional qRT-PCR (Figure S1C) and we found *miR-106b*, *miR-93*, and *miR-25* precursor species to be concordantly expressed in the tumors [r(106b/93) = 0.93; r(106b/25) = 0.78; r(93/25) = 0.88; Table S5]. Of the five tumors overexpressing *miR-106b-25* precursors,



A

	Median Fold-change
miR-1	3.0
miR-155	2.5
miR-205	0.1
miR-203	0.1
miR-202	0.3
miR-20	0.4
miR-26b	0.4

B

	Median Fold-change
miR-21	4.5
miR-223	4.2
miR-25	**3.8**
miR-17-5p	3.7
miR-125b	3.6
miR-181b	3.4
miR-106a	2.8
miR-107	2.7
miR-92	2.5
miR-103	2.5
miR-221	2.4
miR-93	**2.3**
miR-100	2.1
miR-106b	**2.0**
miR-136	0.6
miR-218	0.6
miR-212	0.6
miR-96	0.7
miR-339	0.7

Figure 1. Alteration of miRNA Expression in Chronic Gastritis and Gastric Adenocarcinoma

(A and B) miRNAs significantly associated with either chronic gastritis (A) or gastric adenocarcinoma (B) by SAM analysis (FDR = 0%, q = 0). Red and green colors indicate upregulation and downregulation, respectively. Representative histological features of normal gastric mucosa, chronic gastritis, and gastric adenocarcinoma are shown with hematoxylin & eosin (H&E) staining.

(C) Schematic representation of the *miR-106b-25* cluster genomic locus hosted in the intron 13 of *Mcm7*. The primary transcript of this gene contains all the three miRNAs fused into a unique molecule that we retrotranscribed, amplified, and sequenced from Snu-16 cells using two different sets of primers (#1 and #2).

(D and E) This molecule is not just a byproduct of *Mcm7* transcription, as downregulation of Drosha by RNAi (D) induced a dramatic accumulation of this transcript three tumors also expressed high levels of mature *miR-106b*, *miR-93*, and *miR-25* whereas the remaining tumors displayed variable expression of each mature miRNA, suggesting an additional level of posttranscriptional regulation controlling individual miRNAs.

Mcm7 mRNA was also overexpressed in 5/10 tumors, showing an almost perfect correlation with *miR-106b*, *miR-93*, and *miR-25* precursor levels (r = 0.98, 0.92, 0.72, respectively; Figure S1C and Table S5).

Taken together, these data argue that *miR-106b-25* precursors are specifically overexpressed in a subset of gastric primary tumors in parallel with *Mcm7* mRNA. Although we cannot exclude the possibility of a *miR-106b-25*-independent promoter, our results strongly suggest that *miR-106b-25* transcription in gastric tumors is driven by its host gene, *Mcm7*. Moreover, a posttranscriptional mechanism also plays a major role in determining the levels of mature *miR-106b-25*, as recently proposed for other miRNAs (Thomson et al., 2006).

A Negative Feedback Loop Controls E2F1 and *miR-106b-25* Expression

E2F1 is a transcription factor that transactivates a variety of genes involved in chromosomal DNA replication (Johnson and DeGregori, 2006), including *Mcm7* (Suzuki et al., 1998; Arata et al., 2000). Therefore, we hypothesized that *miR-106b-25* transcription may be similarly regulated by E2F1. To test this hypothesis, we first determined whether endogenous fluctuations in E2F1 protein levels corresponded to similar changes in *Mcm7* and *miR-106b-25* expression. Interestingly, AGS gastric cancer cells, arrested in mitosis by nocodazole treatment for 12 hr, did not express E2F1 protein and showed reduction in *Mcm7* transcript (2-fold) and *miR-106b*, *miR-93*, and *miR-25* precursors (4.0-, 5.2-, and 12.0-fold, respectively), compared to exponentially growing cells. As cells were released and re-entered the G1 phase, E2F1 expression paralleled *Mcm7*, *miR-106b*, *miR-93*, and *miR-25* precursor RNA reaccumulation. (Figures 2A–2C). This process was directly associated with E2F1 expression because its specific overexpression by adenoviral transduction (Figure 2D) or silencing by RNA interference (Figure 2E) also induced consistent changes in *miR-106b-25* precursor levels. Importantly, E2F1 loss of function impacted the expression of mature miRNAs after 72 hr as well (Figure 2F).

To further validate our data in vivo, we analyzed E2F1 protein expression in ten primary gastric tumors by western blot, and we found a positive correlation between E2F1 protein and *Mcm7*/*miR-106b-25* precursor expression (Figure 2G). In fact, four out of five tumors overexpressing E2F1 displayed higher levels of *Mcm7* and *miR-106b-25* precursors (Figure S1C). Of these, three tumors also overexpressed mature *miR-106b*, *miR-93*, and *miR-25* (Figure S1B). However, one tumor showed *Mcm7* and *miR-106b-25* precursor upregulation without detectable levels of E2F1, suggesting that other transcription factors are also involved in the regulation of *miR-106b-25*.

These results indicate that E2F1 regulates *miR-106b-25* expression in parallel with *Mcm7*, supporting the hypothesis that overexpression of these miRNAs in gastric cancer is due, at least in part, to E2F1 upregulation.

Recently, *miR-17-5p* has been proposed as a posttranscriptional regulator of E2F1 (O'Donnell et al., 2005). Given the similarity between *miR-17-5p*, *miR-106b*, and *miR-93* sequences, we explored the possibility that also *miR-106b* and *miR-93* may participate in the regulation of E2F1 expression. Because these miRNAs were diffusely expressed in a panel of 12 gastric cancer cell lines analyzed by qRT-PCR (Figure 3A), we adopted a loss-of-function approach to antagonize *miR-106b-25*. Transfection of LNA antisense oligonucleotides (ASOs) against *miR-106b* and *miR-93* induced an accumulation of E2F1 protein in Snu-16 cells, indicating that endogenous levels of these miRNAs control its expression (Figure 3B). Also, overexpression of these miRNAs by either oligonucleotide transfection or lentiviral transduction (Figure S1D) clearly decreased E2F1 protein levels in Snu-16 and AGS gastric cancer cell lines (Figures 3C and 3D) and inhibited the expression of a reporter vector containing E2F1 3′UTR. Mutation of the predicted miRNA binding sites in the reporter vector abrogated this effect, indicating that *miR-106b* and *miR-93* directly interact with E2F1 3′UTR (Figure 3E). However, E2F1 mRNA decreased by 2-fold upon *miR-106b* and *miR-93* transfection, possibly because of partial mRNA degradation or downmodulation of E2F1 transcriptional activators (Figure 3F).

It has been argued that *miR-17-5p* may secondarily inhibit E2F1 expression by suppressing *AIB-1* protein that in fact activates E2F1 transcription and is also a *miR-17-5p* target (Hossain et al., 2006). While it is very reasonable that miRNAs act on different targets within the same pathway, we analyzed *AIB-1* protein levels in AGS and Snu-16 cells and found a slight decrease or no difference at all in cells transfected with either *miR-106b* or *miR-93*, respectively, suggesting that *AIB-1* is a bona fide low-affinity target of *miR-106b* that may only partially contribute to E2F1 downregulation (Figure 3C).

Together, these results show that E2F1 regulates *miR-106b-25* expression but is also a target of *miR-106b* and *miR-93*, establishing a negative feedback loop in gastric cancer cells. Because E2F1 is known to self-activate its own promoter through a positive feedback loop, these miRNAs may control the rate of E2F1 protein synthesis, preventing its excessive accumulation, as recently proposed for homolog *miR-17-5p* and *miR-20a* (Sylvestre et al., 2007; Woods et al., 2007).

miR-106b and *miR-93* Impair TGFβ-Induced Cell-Cycle Arrest

Our results thus far indicate that *miR-106b-25* transcription is promptly induced by E2F1 as cells exit mitosis and re-enter the G1 phase. On this basis, we hypothesized a possible role for *miR-106b-25* in repressing G0/G1-associated activities, ideally cooperating with E2F1. So, we interrogated the TargetScan database looking for genes known to be negatively regulated by E2F1, and we identified CDKN1A (p21) as a putative target of *miR-106b* and *miR-93.* This gene, frequently dysfunctional in human cancer, is a key inhibitor of the cell cycle (Mattioli et al.,

(E), confirming the presence of an active preliminary miRNA. Bars indicate RNA expression normalized to U6 ± SD. This cluster shares a high degree of homology with *miR-17-92* and *miR-106a-92* clusters, located on chromosomes 13 and X, respectively. Colors identify miRNAs of the same family.

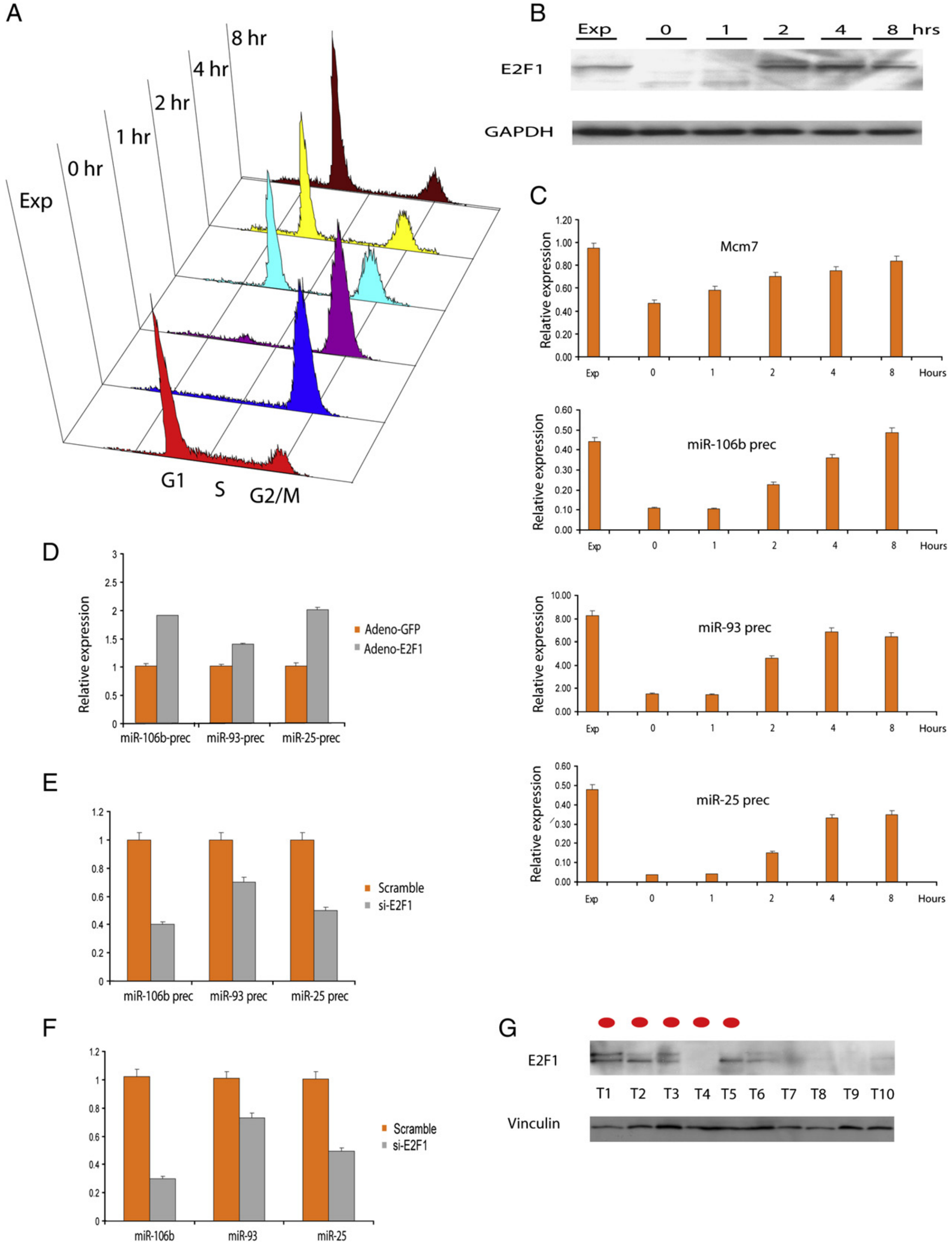


Figure 2. E2F1 Regulates *miR-106b-25* Expression

(A–C) (A) FACS analysis of AGS cells synchronized in mitosis by nocodazole treatment for 12 hr and subsequently released in fresh medium. Cells were harvested at different time points and analyzed for E2F1 protein content by western blot (B) and *Mcm7*, *miR-106b*, *miR-93*, and *miR-25* precursor RNA levels by qRT-PCR

2007). Intriguingly, we confirmed that *miR-106b* and *miR-93* endogenously expressed in Snu-16 cells posttranscriptionally regulate p21. In fact, their inhibition by ASOs enhanced the expression of p21 protein (Figure 4A). Conversely, upregulation of *miR-106b* and *miR-93* achieved by either oligonucleotide transfection (Figure 4B) or lentiviral transduction (Figure 4C) repressed p21 protein expression without significant changes in p21 mRNA levels (Figure 4D). Moreover, *miR-106b* and *miR-93* mimics inhibited the expression of a reporter vector containing p21 3′UTR, while mutation of the predicted miRNA-binding site abrogated this effect (Figure 4E).

Given the importance of p21 in the regulation of cell cycle, we decided to address the role of *miR-106b-25* in controlling the proliferation of gastric cancer cells. Unexpectedly, loss of *miR-106b*, *miR-93*, and/or *miR-25* function induced by ASO transfection did not produce any significant alterations in the cell cycle and proliferation of Snu-16 cells (Figures S2A and S2C). Similarly, overexpression of the three miRNAs by either oligonucleotide transfection or lentiviral transduction did not significantly modify the proliferation rate and colony formation efficiency of AGS cells, although we noticed limited but reproducible cell-cycle perturbations upon *miR-93* overexpression (+8% of cells in S phase; Figures S2B, S2D, and S2E). We obtained similar results using GTL-16 and MKN-74 gastric cancer cell lines (data not shown), indicating that *miR-106b-25* function is not essential for the survival and the proliferation of gastric cancer cells in vitro. However, specific silencing of either p21 or E2F1 by RNAi produced no significant alterations in the proliferation as well (Figures S2G and S2H), confirming that these cancer cell lines are not responsive to p21 basal levels and can well compensate for the loss of E2F1 expression.

Therefore, we decided to address the role of *miR-106b-25* in the presence of TGFβ: this cytokine, by inducing the expression of p21 and other antiproliferative molecules, ensures timely coordinated cell-cycle arrest and apoptosis of mature cells in the gastrointestinal tract, thus controlling the physiological turnover of epithelial cells (van den Brink and Offerhaus, 2007). Impairment of this crucial tumor suppressor pathway is a hallmark of gastric cancer (Ju et al., 2003; Park et al., 1994). However, Snu-16 cells are among the few gastric cancer cell lines still responding to relatively high doses of TGFβ in vitro, undergoing G1/S arrest and subsequent massive apoptosis (Ohgushi et al., 2005 and Figure 5A). Nevertheless, cell viability decreases after 24 hr, thus opening a window to study early molecular changes associated with TGFβ.

Interestingly, stimulation with TGFβ induced marked downregulation of E2F1 protein, *Mcm7* mRNA, and *miR-106b-25* precursors after 16 hr, when cells physiologically undergo G1/S arrest, suggesting that downmodulation of these miRNAs is part of the physiological response to TGFβ (Figures 5B and 5C). To establish the importance of this process, we counteracted *miR-106b-25* downregulation by introducing *miR-106b*, *miR-93*, and/or *miR-25* mimics in Snu-16 cells in the presence of TGFβ. Notably, overexpression of *miR-93* completely abrogated TGFβ-induced cell-cycle arrest, while *miR-106b* partially decreased it ($p < 0.0002$), consistent with the degree of p21 downregulation induced by these miRNAs (Figure 5D). Conversely, antagonizing endogenous *miR-106b* and *miR-93* expression by ASOs significantly increased the number of Snu-16 cells undergoing TGFβ-dependent cell-cycle arrest ($p < 0.0013$) and restored sensitivity to suboptimal doses of TGFβ ($p < 0.0001$), to which these cells are otherwise resistant (Figures 6A and 6B). Accordingly, the degree of p21 upregulation achieved by inhibiting endogenous *miR-106b* and *miR-93* in the presence of TGFβ (Figure 6C) was double than that in basal conditions (Figure 4A), probably supported by the active transcription of p21 mRNA (Figure 6D).

To establish the role of p21 in inducing the phenotype associated with *miR-106b* and *miR-93* gain/loss of function, we specifically silenced p21 by RNAi (si-p21) in Snu-16 cells treated with TGFβ. This recapitulated almost in full the effect of *miR-106b* and *miR-93* overexpression on cell-cycle distribution (Figure 5D), whereas cotransfection of si-p21 with *miR-106b* and *miR-93* dramatically reduced the effect of these miRNAs on TGFβ-induced cell-cycle arrest, suggesting that p21 is a primary target in this biological context (Figure 6E). However, a small but statistically significant effect on TGFβ-dependent cell-cycle arrest by *miR-93* was still observable in the absence of p21 ($p = 0.0272$), implying that other direct or indirect targets cooperate with p21. Analysis of expression for genes involved in the G1/S checkpoint points at p27 as a possible indirect target of *miR-93* (Figure 6F).

From these data we conclude that *miR-106b* and *miR-93* interfere with TGFβ-induced cell-cycle arrest, mainly inhibiting the expression of p21 at the posttranscriptional level. However, p21-independent pathways may be also involved in delivering the complete effect of *miR-93* on cell-cycle control.

miR-25 Cooperates with *miR-106b* and *miR-93* in Preventing the Onset of TGFβ-Induced Apoptosis

Our results so far support a role for *miR-106b* and *miR-93* in modulating the cell-cycle arrest in the early phase of TGFβ stimulation. At this point, we decided to analyze *miR-106b-25* function upon prolonged exposure to TGFβ that eventually results in apoptosis (Ohgushi et al., 2005, and Figure 5B).

To this aim, we examined the viability of Snu-16 cells stimulated with TGFβ for 24–48 hr by tetrazolium reduction assay. Interestingly, introduction of *miR-106b*, *miR-93*, and/or *miR-25* mimics in these cells induced marked resistance to TGFβ (Figure 7A). Conversely, ASO transfection induced a negative trend in the number of viable cells that reached statistical significance ($p = 0.003$) when all the three miRNAs were inhibited at

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(C). Each analysis was performed in triplicate. Bars indicate RNA expression normalized to U6 ± SD.

(D) AGS cells were plated at 90% confluence and starved in 0.5% FBS RPMI 1640 medium for 36 hr. Cells were then infected with either adeno-GFP or adeno-E2F1 viruses at an moi of 25 and incubated for an additional 21 hr: at this time, cells displayed no signs of apoptosis, as determined by morphology, trypan-blue staining, and analysis of subdiploid DNA content (data not shown). *miR-106b*, *miR-93*, and *miR-25* precursors were measured by qRT-PCR as above.

(E and F) Snu-16 cells were transfected with a siRNA against E2F1 (100 nM), and expression of miR-106b-25 precursor (E) and mature (F) species was determined after 72 hr by qRT-PCR, as above. Bars indicate RNA expression normalized to U6 ± SD.

(G) Expression of E2F1 protein in the same gastric primary tumors presented in Figure S1. Red circles indicate overexpression of *Mcm7* and *miR-106b-25* precursor RNA in the corresponding tumors, as determined by qRT-PCR.

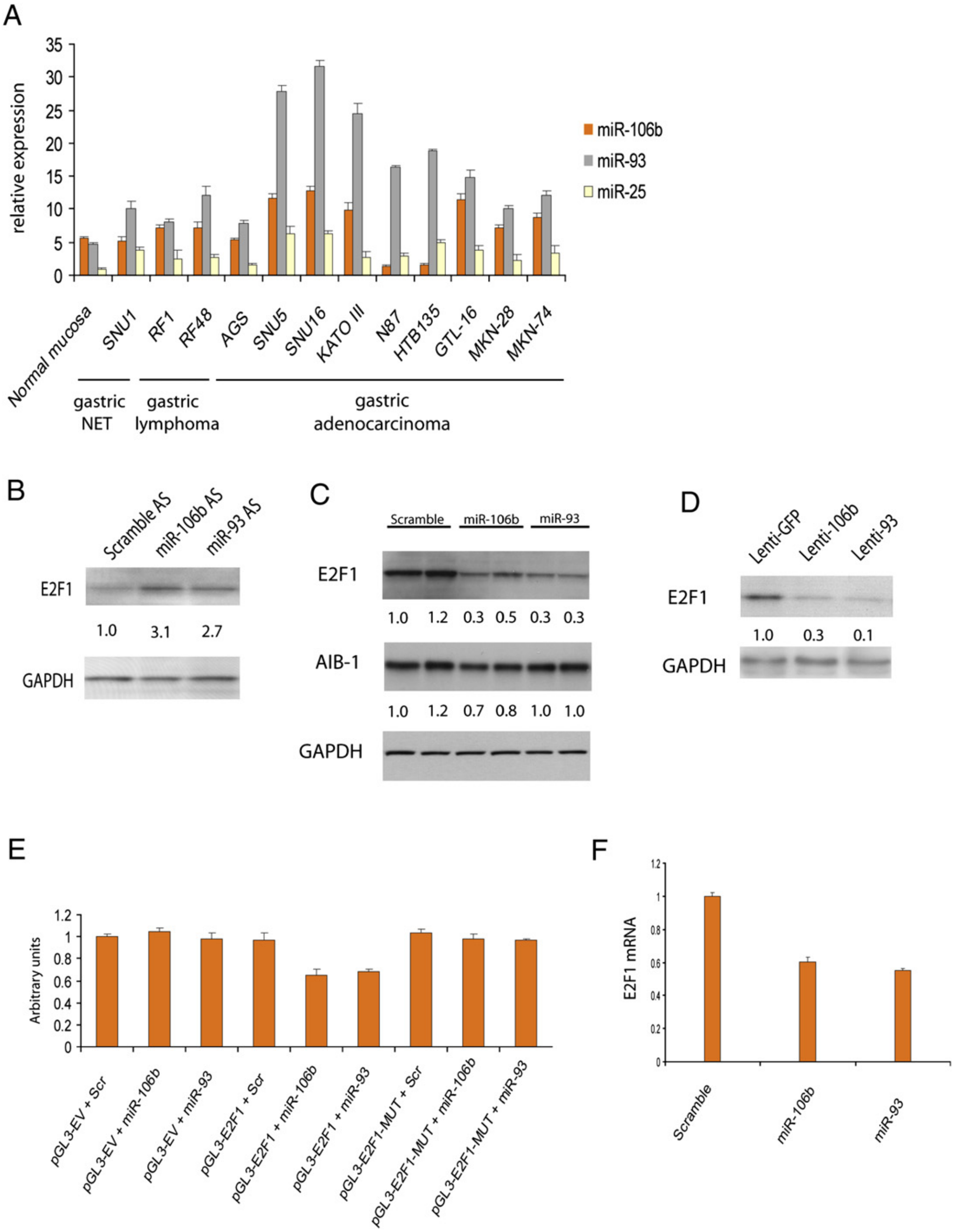

Figure 3. E2F1 Is a Target of *miR-106b* and *miR-93*

(A) Endogenous expression of mature *miR-106b*, *miR-93*, and *miR-25* in human gastric cancer cell lines and normal mucosa determined by stem-loop qRT-PCR; bars indicate RNA expression normalized to U6 ± SD. Snu-1 cells are thought to derive from a gastric neuroendocrine tumor (NET), while RF1 and RF48 cells are from a B cell lymphoma of the stomach. All the other cell lines are from gastric adenocarcinoma.

(B–D) Western blot of Snu-16 cells 48 hr after inhibition of *miR-106b* and *miR-93* by ASO transfection (B) or overexpression of the same miRNAs by oligonucleotide transfection (C) or lentiviral transduction (D). Scramble RNA or LNA oligonucleotides were used as negative control. Protein expression was quantified and normalized to GAPDH. Similar results were obtained in AGS and MKN-74 cells (data not shown).

(E) Luciferase assay showing decreased luciferase activity in cells cotransfected with pGL3-E2F1-3′UTR and *miR-106b* or *miR-93* oligonucleotides. Deletion of the first three bases in three putative *miR-106b*/*miR-93* binding sites, complementary to miRNA seed regions, abrogates this effect (MUT). Bars indicate Firefly luciferase activity normalized to Renilla luciferase activity ± SD. Each reporter plasmid was transfected at least twice (on different days), and each sample was assayed in triplicate.

(F) qRT-PCR analysis showing E2F1 mRNA downregulation in the same cells presented in (C). Bars indicate RNA expression normalized to U6 ± SD.

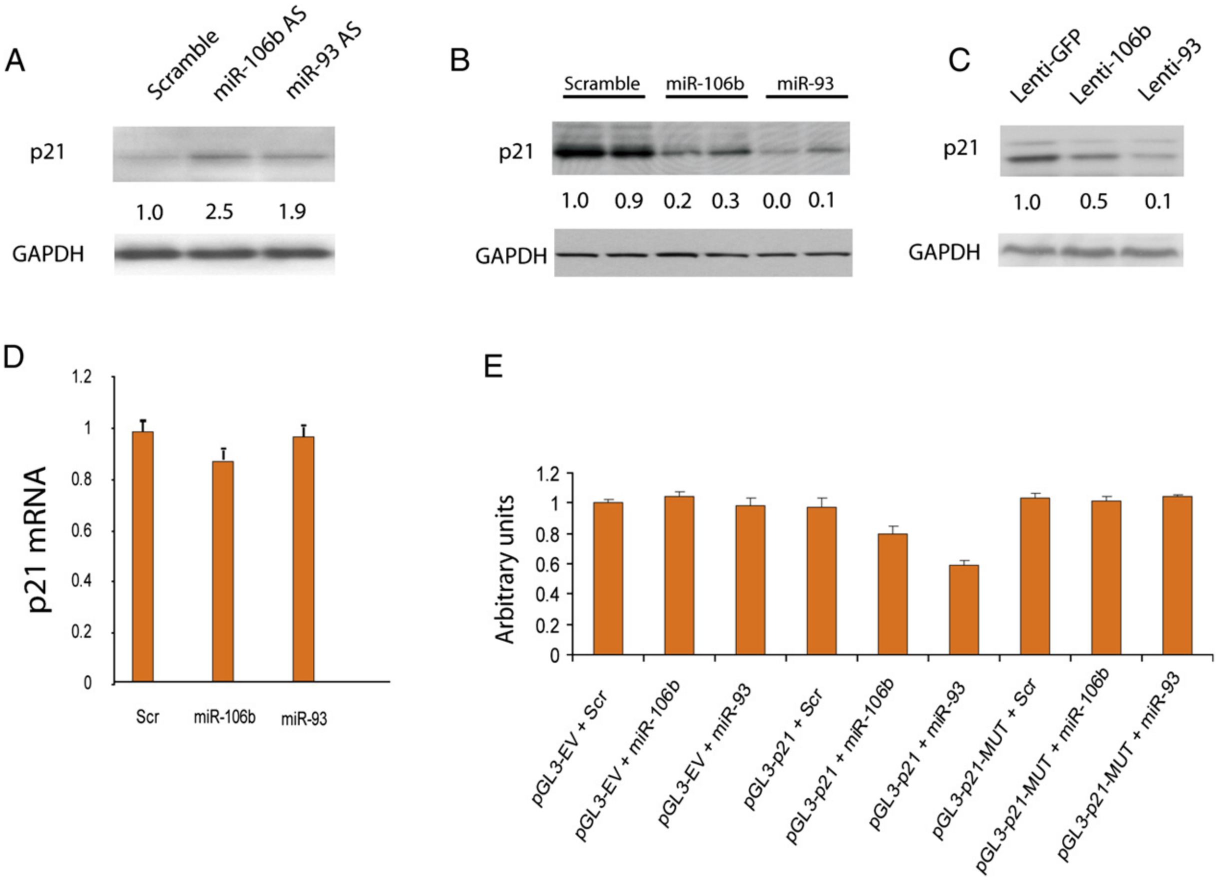

Figure 4. *miR-106b* and *miR-93* Repress p21 Protein Expression

(A–C) P21 expression in Snu-16 cells grown in 0.5% FBS RPMI 1640 after transfection with either *miR-106b* and *miR-93* ASOs (A) or mimics (B) or upon lentiviral transduction of the same miRNAs (C).

(D) qRT-PCR results showing no significant difference in p21 mRNA levels in Snu-16 cells transfected with either *miR-106b* or *miR-93* oligonucleotides. Bars indicate RNA expression normalized to U6 ± SD.

(E) Reporter assay showing decreased luciferase activity in cells cotransfected with pGL3-p21-3′UTR and *miR-106b* or *miR-93* oligonucleotides. Deletion of the first three bases of *miR-106b*/*miR-93* predicted binding site, complementary to miRNA seed regions, abrogates this effect (MUT). Bars indicate Firefly luciferase activity normalized to Renilla luciferase activity ± SD. Each reporter plasmid was transfected at least twice (on different days), and each sample was assayed in triplicate.

the same time (Figure 7B). This result was confirmed by FACS analysis that showed a significant increase in the number of subdiploid cells upon silencing of the three miRNAs ($p < 0.001$). Moreover, the higher sensitivity of this assay allowed detection of smaller but significant changes ($p < 0.001$) in the percentage of subdiploid cells upon individual inhibition of *miR-106b*, *miR-93*, or *miR-25* (Figure 7C). Finally, silencing of *miR-106b-25* partially restored sensitivity to TGFβ in otherwise resistant MKN-74 cells (Figure S3). Together, these results are consistent with a model where endogenous *miR-106b*, *miR-93*, and *miR-25* cooperate in modulating the expression of one or more targets mediating TGFβ-dependent apoptosis.

Thus, we searched TargetScan database looking for effectors of apoptosis, and we identified *BCL2L11* (*Bim*) as the only strong candidate out of 18 human genes harboring putative binding sites for *miR-106b*, *miR-93*, and *miR-25* at the same time (Table S6). *Bim* is a BH3-only protein that critically regulates apoptosis in a variety of tissues by activating proapoptotic molecules like *Bax* and *Bad* and antagonizing antiapoptotic molecules like *Bcl2* and *BclXL* (Gross et al., 1999). A fine balance in the intracellular concentrations of *Bim* and its partner proteins is crucial in order to properly regulate apoptosis. As a matter of fact, *Bim* is haploinsufficient, and inactivation of even a single allele accelerates Myc-induced development of tumors in mice without loss of the other allele (Egle et al., 2004). Notably, *Bim* is the most downstream apoptotic effector of the TGFβ pathway, and its downmodulation abrogates TGFβ-dependent apoptosis in Snu-16 cells (Ohgushi et al., 2005).

Thus, we wanted to verify whether *Bim* was a direct target of *miR-106b-25*. Snu-16 cells express all the three major isoforms of *Bim*, namely *Bim EL*, *Bim L*, and *Bim S*. Intriguingly, antagonizing endogenous *miR-25* by ASO transfection induced an accumulation of all the three isoforms in Snu-16 cells, whereas *miR-25* overexpression by either oligonucleotide transfection or lentiviral transduction reduced their expression. On the contrary, *miR-106b* and *miR-93* did not influence Bim expression in three out of three tested gastric cancer cell lines (Figure 7D).

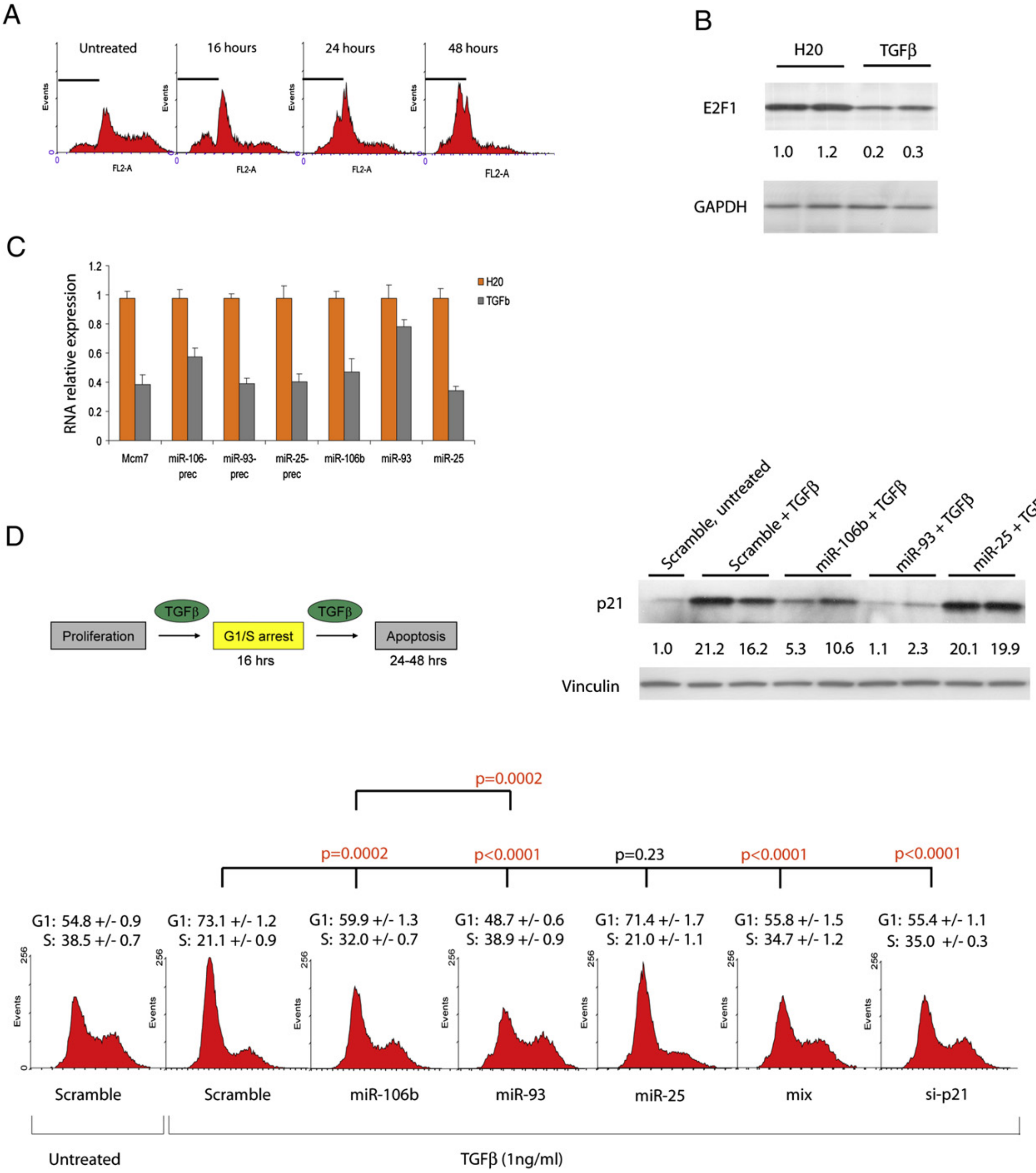

Figure 5. Overexpression of *miR-106b* and *miR-93* Interfere with TGFβ-Dependent G1/S Cell-Cycle Arrest

(A) Physiological response of Snu-16 cells to 1 ng/ml TGFβ: in the early phases of stimulation (16 hr), cells undergo a G1/S cell-cycle arrest, while apoptosis is still limited, as determined by subdiploid DNA content. The number of cells undergoing apoptosis progressively increases in the following hours.

(B and C) Downregulation of E2F1 protein (B) and *Mcm7*, *miR-106b*, *miR-93*, and *miR-25* precursors (C) 16 hr after TGFβ stimulation. Bars indicate RNA expression normalized to U6 ± SD.

(D) Snu-16 cells were transfected with the indicated oligonucleotides and treated with 1 ng/ml TGFβ after 12 hr. (Upper panel) p21 protein expression. (Bottom panel) FACS analysis, comparison of G1/S fractions between mock- and miRNA-transfected cells using unpaired Student's t test.

While it is still possible that *miR-106b* and *miR-93* cooperate with *miR-25* in regulating *Bim* expression in other tissues, this supports a model where multiple effectors of apoptosis are coordinately repressed by each of the three miRNAs in gastric cancer.

Therefore, we decided to focus on *Bim* as one of these apoptotic effectors, and we determined that *miR-25* predicted binding sites on its 3′UTR mediate target recognition and subsequent inhibition of translation by luciferase assay (Figure 7E). Moreover, *Bim EL* and *Bim L* mRNA levels were unchanged in Snu-16 cells upon *miR-25* overexpression, which is indicative of a posttranscriptional regulatory mechanism (Figure 7F).

In order to establish the importance of *Bim* downregulation relative to *miR-25*-specific antiapoptotic function, we suppressed *Bim* protein in Snu-16 cells using a siRNA against its three major isoforms (si-Bim, Figure 7D), and we subsequently treated these cells with TGFβ for 24 hr. Notably, protection from apoptosis conferred by si-Bim and *miR-25* was very similar, as determined by subdiploid DNA content and Annexin V staining. Moreover, cotransfection of *Bim* and *miR-25* did not have significant additive effects ($p = 0.6328$), suggesting that *Bim* downregulation is a main mechanism of resistance to TGFβ-induced apoptosis in *miR-25*-overexpressing cells (Figure 7G and Figure S4).

In conclusion, we provide evidence that *miR-106b-25* cluster, activated by E2F1 and upregulated in human gastric adenocarcinomas, alters the physiological response of gastric cancer cells to TGFβ, affecting both cell-cycle arrest and apoptosis (Figure 8). These findings are of particular relevance in a gastric cancer model, as impairment of the TGFβ tumor suppressor pathway is a critical step in the development of gastric tumors.

DISCUSSION

In this study we performed a genome-wide analysis of miRNA expression in different steps of gastric carcinogenesis. Since the vast majority of gastric tumors originate from a chronic inflammatory background (Uemura et al., 2001), we considered of particular relevance discriminating between preneoplastic and tumor-specific alterations. Here, we identified the specific overexpression of a miRNA cluster in human tumors that had been ignored thus far. Although we focused on gastric cancer, overexpression of *miR-106b*, *miR-93*, and *miR-25* in other types of cancer may be a common, yet underestimated, event. In fact, *miR-106b-25* expression is intimately linked with the expression of E2F1 and *Mcm7* that is involved in basic mechanisms of cellular proliferation. For example, *Mcm7* is frequently overexpressed in prostate cancer (Ren et al., 2006), and in fact, we previously described *miR-25* upregulation in a large-scale miRNA study on this type of cancer (Volinia et al., 2006). Moreover, we showed that stem-loop qRT-PCR probes commonly used in assaying the expression of *miR-92*, which is overexpressed in most human tumors (Volinia et al., 2006), cross-hybridize with *miR-25*. However, given the nearly identical sequences, it is very likely that *miR-106b-25* and *miR-17-92* cooperate in exerting similar, if not identical, functions: in fact, we found that *miR-17-5p*, *miR-18a*, and *miR-20a* also inhibit *p21* expression, whereas *miR-92* represses *Bim* expression (F.P. and A.V., unpublished data). Moreover, both *miR-106b-25* and *miR-17-92* are regulated by E2F1. These clusters also exhibit some differences, though. For example, *miR-106b* resembles *miR-17-5p* but it is three nucleotides shorter: it has been reported that specific sequences in the 3′ termini can define the intracellular localization of miRNAs (Hwang et al., 2007). Moreover, the *miR-19* family is not represented in the *miR-106b-25* cluster (Figure 2A).

On the other hand, *miR-93* belongs to the same family of *miR-372* and *miR-373*: these miRNAs are overexpressed in testicular germ cell tumors where they impair *LATS2* expression, making cells insensitive to high *p21* levels (Voorhoeve et al., 2006). In our study, *miR-93* acts within the same pathway, directly targeting *p21* expression. Therefore, this family of miRNAs seems to be involved in the control of a crucial hub for the regulation of cell cycle and may have particular relevance in cancer. Moreover, *miR-93* shares high sequence homology with *miR-291-3p*, *miR-294*, and *miR-295*: these miRNAs are specifically expressed in pluripotent ES cells, and they are either silenced or downregulated upon differentiation (Houbaviy et al., 2003). Given our results, we speculate that these miRNAs may be similarly involved in the regulation of *p21*.

From this and previous studies, it is becoming clear that miRNAs play a role in the control of cell cycle through different mechanisms. In the case of E2F1, miRNAs seem to act mainly in the context of regulatory, redundant feedback loops. In fact, *miR-106b*, *miR-93*, *miR-17-5p*, and miR-20a, located on separate miRNA clusters, are regulated by E2F1 and presumably cooperate in inhibiting its translation. Whether miRNAs are essential in determining temporal regulation of E2F1 expression is still unclear and deserves further studies.

At the same time, we found these miRNAs to be involved in the control of *p21* expression and early response to TGFβ. Although we focused on the TGFβ tumor suppressor pathway, it is conceivable that they also control other tumor suppressor pathways converging on *p21*. Loss of *p21* function by mutation, deletion, hypermethylation, ubiquitination, or mislocalization is a frequent event and a negative prognostic factor in human gastric cancer (Mattioli et al., 2007). However, the role of miRNAs in *p21* regulation has not yet been reported. Since 80% of our gastric primary tumors did not express *p21* protein at detectable levels, we could not establish an inverse correlation between miRNAs and *p21* protein expression. However, *p21* mRNA levels in primary tumors were often comparable to normal tissues, indicating posttranscriptional regulation as a frequent cause of *p21* downregulation in gastric cancer (F.P and A.V., unpublished data).

Interestingly, induction of p21 expression seemed to be a prerequisite to elicit a *miR-106b*/*miR-93*-associated response in the early phase of TGFβ stimulation. Conversely, silencing p21 by RNAi dramatically decreased the effect of these miRNAs on cell cycle. Although hundreds of different targets are predicted for each miRNA by computational methods, there is increasing evidence that ‘‘primary miRNA targets’’ may be critical for specific biological functions. For example, *miR-10b* enhances cell motility and invasiveness of breast cancer cells, but this phenotype is completely reverted upon constitutive expression of its target *HOXD10*, although over 100 targets are predicted for this miRNA (Ma et al., 2007). Of course, these observations do not exclude other contexts where parallel regulation of multiple targets by a single miRNA is necessary to exert a specific function. Furthermore, it is also conceivable that multiple miRNAs cooperate in exerting the same function.

This is the case of the *miR-106b-25* cluster that protects gastric cancer cells from apoptosis. Such effect is partitioned between the three miRNAs that cooperate in repressing the expression of different proapoptotic molecules. We identified *Bim*, the most downstream apoptotic effector of the TGFβ pathway (Ohgushi et al., 2005), as a key target of *miR-25.* This is of particular relevance in a gastric cancer model. In fact, TGFβ is one of the

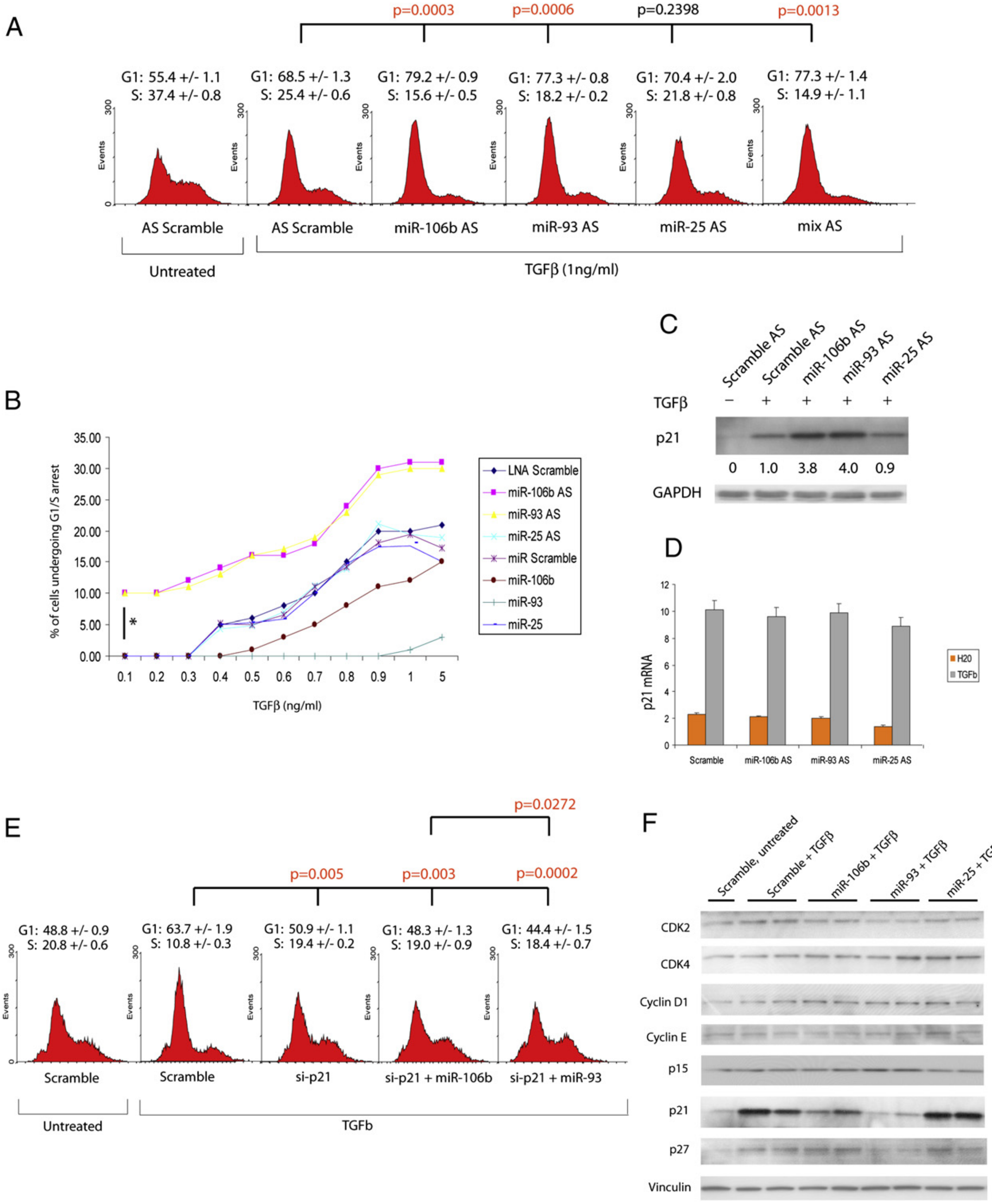

Figure 6. Inhibition of Endogenous *miR-106b* and *miR-93* Expression Enhances TGFβ-Dependent G1/S Cell-Cycle Arrest

(A) Analysis of cell cycle in Snu-16 cells treated with TGFβ upon inhibition of endogenous miRNAs by ASO transfection. p value was calculated comparing the G1 fraction in ASO transfected cells versus mock-transfected cells (unpaired Student's t test).

(B) Dose-response curve of Snu-16 treated with graded doses of TGFβ ranging from 0.1 to 5.0 ng/ml. Inhibition of endogenous *miR-106b* or *miR-93* by ASO transfection restores sensitivity of Snu-16 cells to TGFβ doses to which they are otherwise resistant (0.1–0.3 ng/ml), as determined by FACS analysis. $*p < 0.0001$.

(C and D) Analysis of p21 protein (C) and p21 mRNA expression (D) by western blot and qRT-PCR, respectively. Bars indicate RNA expression normalized to U6 ± SD. The degree of p21 protein upregulation induced by inhibition of endogenous *miR-106b* and *miR-93* is greatly enhanced by the presence of TGFβ, possibly supported by the increased transcription of p21 mRNA.

main regulators of gastric homeostasis and is essential in regulating the physiological turnover of epithelial cells through apoptosis (van den Brink and Offerhaus, 2007). While the identity of *miR-106b* and *miR-93* proapoptotic targets remains elusive, we could clearly detect antiapoptotic and proapoptotic responses associated with *miR-106b*, *miR-93*, and/or *miR-25* overexpression and inhibition, respectively; these properties emerge in the late phase of TGFβ stimulation when cell-cycle arrest is revoked and apoptosis becomes the dominant process characterizing the response of gastric cells to TGFβ. The small but significant alterations observed upon inhibition of single miRNAs, readily detected by analysis of subdiploid DNA content, acquire biological consistency when the three ASOs are delivered together, confirming the cooperative relationship between these clustered miRNAs.

Although a negative trend was observed in TGFβ-stimulated cells transfected with single ASOs by both tetrazolium reduction assay and analysis of subdiploid DNA content, this did not reach statistical significance in the tetrazolium reduction assay. This is to be imputed to the 5%–10% standard error associated with this assay that statistically excludes smaller differences. On the contrary, the standard error in the analysis of subdiploid DNA content was below 2% in our hands.

When we looked at *Bim* expression in primary tumors, we noticed general overexpression compared to normal tissues (F.P. and A.V., unpublished data). This is consistent with previous studies showing that *Bim* is induced by oncogenic stress as a safeguard mechanism to prevent aberrant proliferation. Specifically, *Bim* is overexpressed in Myc transgenic mice, determining extensive apoptosis of normal cells. However, the onset of tumors in these mice coincides with the loss of one *Bim* allele that becomes insufficient. Still, *Bim* remains definitely overexpressed in tumors compared to healthy tissues that are not subject to oncogenic stress (Egle et al., 2004). Therefore, it is hard to define a threshold below which *Bim* insufficiency occurs, and alternative strategies are needed to define the importance of *miR-25* upregulation in vivo.

Several mechanisms have been described leading to *Bim* downregulation in cancer, from transcriptional regulation to protein degradation (Yano et al., 2006; Tan et al., 2005). While all of these mechanisms clearly contribute to *Bim* silencing, we propose *miR-25* interference as an additional mechanism of *Bim* posttranscriptional regulation in gastric cancer.

It has been extensively debated whether miRNAs are just fine-tuning molecules or they act as key gene switches. Recent studies suggest that both hypotheses are probably true, depending on the specific biological context. From this perspective, the therapeutic potential of miRNAs in cancer may be strictly associated with the occurrence of specific miRNA-dependent functional alterations. Knowing the mechanisms of action of tumor-related miRNAs will be essential in one day establishing the molecular diagnosis of miRNA-dependent tumors, allowing the rational selection of those patients eventually responding to miRNA-based therapies.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments

All cell lines were obtained by ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Cells were transfected with Lipofectamine 2000 (Invitrogen) using 100 nM miRNA precursors (Ambion), 100 nM si-p21 (Santa Cruz), 100 nM si-Bim (Cell Signaling), or 100 nM LNA miRNA antisense oligonucleotides (Exiqon). Protein lysates and total RNA were collected at the time indicated. miRNA processing and expression were verified by northern blot and stem-loop qRT-PCR. We confirmed transfection efficiency (>95%) using BLOCK-IT Fluorescent Oligo (Invitrogen) for all the cell lines.

For synchronization experiments, AGS cells were grown in 10% FBS RPMI 1640 containing 0.03 μg/ml nocodazole for 12 hr and subsequently released in fresh medium. Progression through the cell cycle was followed by FACS analysis until 8 hr, after which cells rapidly lost synchronization.

For TGFβ experiments, 2×10^6 Snu-16 cells were transfected in 6-well plates in a 1:1 mixture of Optimem (GIBCO) and RPMI 1640 10% FBS (Sigma) using 5 μl Lipofectamine 2000 and 100 nM miRNA precursors (Ambion) or LNA antisense oligonucleotides (Exiqon). After 12 hr, medium was replaced with RPMI 1640 10% FBS containing 1 ng/ml human recombinant TGFβ1 (Sigma). Number of viable cells was assayed using WST tetrazolium salt (CCK-8, Dojindo) as per the manufacturer's instructions. All the experiments were performed in triplicate. Results were expressed as mean ± SD.

qRT-PCR

Mature miRNAs and other mRNAs were assayed using the single-tube TaqMan MicroRNA Assays and the Gene Expression Assays, respectively, in accordance with manufacturer's instructions (Applied Biosystems, Foster City, CA). All RT reactions, including no-template controls and RT minus controls, were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems). RNA concentrations were determined with a NanoDrop (NanoDrop Technologies, Inc.). Samples were normalized to RNU49 or CAPN2 (Applied Biosystems), as indicated. Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Applied Biosystems). Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative Ct method.

Luciferase Assays

MKN-74 gastric cancer cells were cotransfected in six-well plates with 1 μg of pGL3 firefly luciferase reporter vector (see Supplemental Experimental Procedures), 0.1 μg of the phRL-SV40 control vector (Promega), and 100 nM miRNA precursors (Ambion) using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured consecutively by using the Dual Luciferase Assay (Promega) 24 hr after transfection. Each reporter plasmid was transfected at least twice (on different days) and each sample was assayed in triplicate.

Flow Cytometry

For cell-cycle analysis, 2×10^6 cells were fixed in cold methanol, RNase-treated, and stained with propidium iodide (Sigma). Cells were analyzed for DNA content by EPICS-XL scan (Beckman Coulter) by using doublet discrimination gating. All analyses were performed in triplicate and 20,000 gated events/sample were counted. For apoptosis analysis, cells were washed in cold PBS, incubated with Annexin V-FITC (BD Biopharmingen) and PI (Sigma) for 15 min in the dark, and analyzed within 1 hr.

Statistical Analysis

Results of experiments are expressed as mean ± SD. Student's unpaired t test was used to compare values of test and control samples. $p < 0.05$ indicated significant difference.

(E) Snu-16 cells were transfected with a siRNA against p21 alone or in combination with either *miR-106b* or *miR-93* mimics and treated with 1 ng/ml TGFβ for 16 hr. While *miR-106b* lost all of its effect on cell cycle, *miR-93* still maintained a residual effect after p21 silencing. This differential response between *miR-106b* and *miR-93* is statistically significant (p = 0.0272).

(F) Analysis of expression by western blot of various proteins involved in the G1/S checkpoint upon TGFβ stimulation.

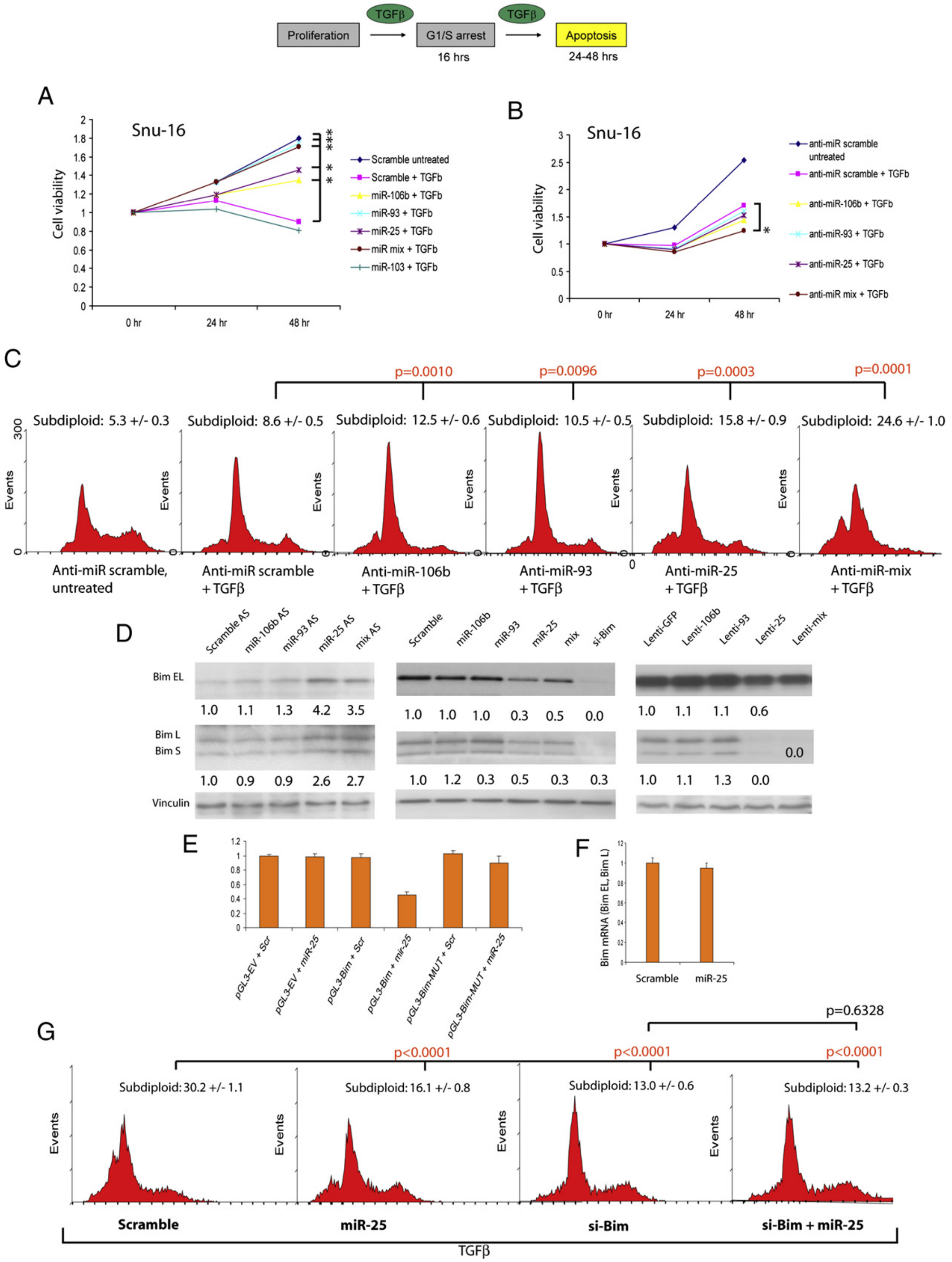

Figure 7. ***miR-25*** **Cooperates with** ***miR-106b*** **and** ***miR-93*** **in Preventing the Onset of TGFβ-Induced Apoptosis**

(A) CCK-8 viability assay of Snu-16 cells transfected with miRNA mimics. Asterisk indicates significant difference ($p < 0.001$) in the number of viable cells upon transfection of *miR-106b*, *miR-93*, *miR-25*, and/or *miR-106b-25* and subsequently treated with 1 ng/ml TGFβ for 48 hr.

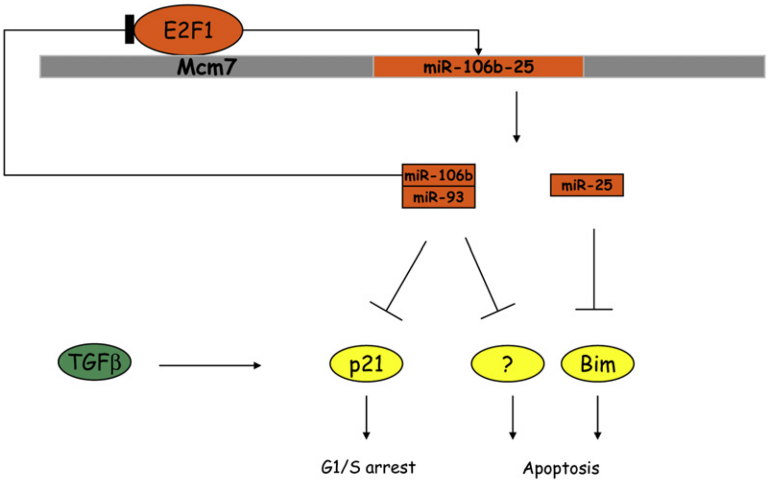

Figure 8. The E2F1/miR-106b-25/p21 Pathway
A model summarizing the mechanism of action of *miR-106*, *miR-93*, and *miR-25* described in this study.

ACCESSION NUMBERS

Microarray data were deposited in the ArrayExpress database (accession number E-TABM-434).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, four supplemental figures, and six supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/3/272/DC1/.

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(B) Conversely, inhibition of *miR-106b*, *miR-93*, and *miR-25* cooperatively augments the response to TGFβ: statistical significance ($p < 0.001$) was reached upon transfection of a mixture of the three ASOs.
(C) Significant loss of viability was confirmed by analysis of subdiploid DNA content.
(D) Bim protein expression in Snu-16 cells at 48 hr posttransfection with either miRNA mimics or ASOs or after lentiviral transduction of the same miRNAs. Same effects on Bim expression were obtained in AGS and MKN-74 cells (data not shown).
(E) Luciferase assay showing decreased luciferase activity in cells cotransfected with pGL3-Bim-3′UTR and *miR-25*. Deletion of the first three bases of *miR-25* predicted binding sites, complementary to miRNA seed regions, abrogates this effect (MUT). Bars indicate Firefly luciferase activity normalized to Renilla luciferase activity ± SD. Each reporter plasmid was transfected at least twice (on different days), and each sample was assayed in triplicate.
(F) qRT-PCR analysis showing no difference in *Bim* mRNA (Taqman probe recognizing the two major isoforms Bim EL and Bim L) in Snu-16 cells transfected with *miR-25* oligonucleotide. Bars indicate RNA expression normalized to U6 ± SD.
(G) FACS analysis of subdiploid DNA content in Snu-16 cells transfected with *miR-25* oligonucleotide, si-Bim, both, or a scramble oligonucleotide and subsequently treated with 1 ng/ml TGFβ for 24 hr. Statistical analysis as above.

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