Synthetic MicroRNA Designed to Target Glioma-Associated Antigen 1 Transcription Factor Inhibits Division and Induces Late Apoptosis in Pancreatic Tumor Cells

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Abstract

Purpose: To determine whether the synthetic microRNAs (miRNA) could effectively target tumor cells we designed several miRNA complementary to glioma-associated antigen-1 (Gli-1) mRNA and investigated their ability to inhibit tumor cell proliferation. The sonic hedgehog pathway is an early and late mediator of tumorigenesis in epithelial cancers. Activation of sonic hedgehog signaling seems to precede transformation of tissue stem cells to cancerous stem cells, with the Gli-1 transcription factor functioning as a mediator of environmental signals. Inhibiting cancer cell proliferation by targeting the Gli-1 effector pathway is difficult to achieve by chemotherapeutic agents or short interfering RNA.

Experimental Design: We hypothesized that targeting the 3′-untranslated region of Gli-1 mRNA would effectively inhibit tumor cell proliferation. To test this hypothesis, we used synthetic miRNAs of our own design and corresponding duplex/small temporal RNAs by introducing three-nucleotide loops in the 3′-untranslated region Gli-1 sequence of high GU content.

Results: We found that miRNA (Gli-1-miRNA-3548) and its corresponding duplex (Duplex-3548) significantly inhibited proliferation of Gli-1$^+$ ovarian (SK-OV-3) and pancreatic (MiaPaCa-2) tumor cells. The miRNAs mediated delayed cell division and activation of late apoptosis in MiaPaCa-2 cells. This is the first demonstration of inhibition of pancreatic tumor cell division by designed miRNA.

Conclusions: Gli-1 miRNAs should significantly add to the general understanding of the mechanisms of metastasis and contribute toward the design of better treatments for epithelial cancers.

Important gene hierarchies coding for components of various signal transduction pathways regulate cell growth and proliferation during development. One such pathway is the sonic hedgehog (Shh)→Patched→release of Patched inhibition of Smoothened→Glioma-associated antigen-1 (Gli-1) pathway (1). Shh signaling is critical to the specification of the cell fate of many tissues during early organogenesis and is mediated by the *Gli* family of transcription factors. One of these factors, the *Gli-1* gene, encodes a transcriptional activator. Two other members, *Gli-2* and *Gli-3*, have been identified. Gli-2 compensates for weak Gli-1 signaling, and Gli-3 reverses the inhibitory effects of Gli-1 or Gli-2 (2–4).

The *Gli-1* oncogene is amplified in cancer cells, sometimes in parallel with HER-2 (5). Constitutive activation of the Hedgehog (Hh)→Gli-1 signaling cascade has recently been implicated in the growth of a number of human malignancies (6). It has been proposed, based on identification of Gli-1 direct target genes, that Hh-driven tumorigenesis relies on promotion of G_1-S phase progression, cell survival, and increase in metastatic potential due to signal-independent division of Gli-1-related cancer cells (6–8). The mechanisms of interaction between signaling pathways have only recently began to be elucidated. A limited amount of information about the interaction of Hh→Wnt signaling is available. It seems that Shh is secreted from differentiated epithelial cells to induce secreted frizzled-related protein-1 (9) expression in mesenchymal cells. Secreted frizzled-related protein 1 antagonizes the effects of canonical Wnt signaling (9). It is interesting to note that several genes that are known to be induced by Shh and/or Wnt contain enhancer elements with conserved Gli sites.

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Therefore, Gli signaling may be involved in integration of Shh and Wnt signals during development (10).

Insulin growth factor-I activation of phosphatidylinositol 3-kinase/AKT potentiates Gli-activation induced by low levels of Shh. Insulin growth factor I alone is insufficient to induce Gli-dependent transcription. Thus, another stimulus is needed. Protein kinase A and glycogen synthase kinase 3β sequentially phosphorylate Gli-2 at multiple sites, resulting in reduction of its transcriptional activity. AKT positively regulates Shh signaling by controlling protein kinase A–mediated Gli inactivation (1). This suggests a synergistic cross-talk between phosphatidylinositol 3-kinase/AKT and Shh signaling in Hh-dependent tumors. These recent studies have investigated only the role of Gli-2 in Hh-mediated signaling. It is unknown whether these conclusions are applicable to Gli-1, or whether Gli-2 functions as a "default" or a redundant pathway that maintains tumor proliferation, when Gli-1 is inhibited. Alternatively, HER-2/neu receptor activation through dimerization with epidermal growth factor receptor (HER-1) and HER-3 can activate the phosphatidylinositol 3-kinase/AKT pathway (11, 12). As a consequence, the expression of Forkhead-transcription actor (FOXO3a), which regulates the estrogen-receptor α (ER-α), is down-regulated by the HER-2→phosphatidylinositol idylinositol 3-kinase/AKT pathway (13).

Except for the products of *Patched* and *WNT* genes, few direct downstream targets of Gli-1 protein are known. Gli-1 proteins bind and up-regulate cyclin D2, implicating that Gli-1 is involved in the regulation of the cell cycle. *Gli-1* genes are important to cell adhesion, movement, and apoptosis, and they up-regulate genes such as osteopontin, which in turn down-regulate expression of plakoglobin (γ-catenin) and embigin (14). The repression of embigin and plakoglobin reduces cell adhesion. Together, these findings indicate a significant role for Gli-1 in accelerated cell division, invasion, and metastasis in cancer cells.

It has been proposed that proliferation of prostate tumor cells can become androgen and Shh independent but not Gli-1 independent. The autonomous activation of the Gli-1/2 pathway provides metastatic tumor cells with the additional advantage of efficient proliferation, away from the epithelium, which continually produces Shh (7). Because Gli function is a last and essential step of the Shh pathway, its inhibition may inhibit Shh signaling at any level (15, 16). Thus, Gli-1/2 pathway–blocking agents might be used to combat epithelial cancers of any grade.

Cyclopamine and other small-molecule inhibitors have been used in animal models to inhibit Smoothened activity in prostate and pancreatic cancer xenografts with good results (17, 18). Small-molecule antagonists of Shh pathway have been developed; some of these molecules are now in clinical trials for basal cell carcinoma, although clinical activity is less than anticipated (18). There is concern for use of cyclopamine in humans because of its toxicity *in vitro*.[6] In humans, currently, only topical cyclopamine has been developed in clinical trials for treatment for psoriasis.

Gene silencing by microRNAs (miRNA) is a natural mechanism, which is highly conserved in evolution, for suppressing specific gene activities. A number of natural miRNAs regulate stem cell division (19, 20). RNA sequences that generate miRNAs that inhibit the expression of regulatory proteins have not yet been described for *Gli* gene transcripts. We engineered Gli-1 miRNA to down-regulate Gli-1 protein expression. The engineered Gli-1 miRNA and Gli-1 small temporal/duplex miRNA (Duplex-3548) significantly inhibited the proliferation and division of pancreatic and ovarian tumor cells. An unexpected effect of the Duplex-3548 was the decrease in the number of tumor cells with high levels of the HER-2 receptor (HER-2^{hi}).

Materials and Methods

Human tumor lines. Panc-1, MiaPaCa-2, HPAC, MOH (pancreatic cancer), PC-3 (prostate cancer), and SK-OV-3 (ovarian cancer) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture in complete RPMI 1640 containing 10% FCS.

Immunofluorescence. Antibodies against Gli-1 and Gli-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies react with Gli-1 and Gli-2 by immunofluorescence (21). There is no specific reference for Gli-2. The anti-Gli-1 antibody was raised against a peptide mapping the COOH terminus of Gli-1 of human origin. The antibody against Gli-2 was raised against a peptide mapping near the NH_2 terminus of Gli-2 of human origin.

All other antibodies used were described (22). To detect and quantitate Gli-1 and Gli-2 proteins, cells were permeabilized, incubated with a blocking antibody, followed by Gli-1- and Gli-2-specific antibodies as described (23). The fluorescence of cells was determined over a range of antibody concentrations. One microgram of anti-Gli-1/2 antibody per 50,000 cells and 0.25 μg secondary antibody-FITC conjugate/50,000 cells resulted in the lowest background staining. The sensitivity of the staining was verified by confocal microscopy (data not shown). The effects of miRNAs on cellularity (tumor cell size) were determined from the forward scatter geometric means values (x^2). Cells of forward scatter >500 were designed as large size whereas cells of forward scatter <500 were designed as intermediate size. The relative density of the Gli-1 protein in tumor was determined from the geometric mean (y^2) of fluorescence intensity of antibody staining.

miRNAs. Design and synthesis of the Gli-1 and HER-2 miRNAs was done as previously described (22). Negative control siRNA was obtained from Dharmacon (Lafayette, CO). As negative control miRNA, we used HER-2-miRNA-4350-14U, which had no effect on SK-OV-3 and other cell lines (22).

Cell proliferation and apoptosis assays. Cells were allowed to attach and proliferate in wells for 48 hours before transfection with the miRNAs. Transfected cells were stimulated with FCS (10% final concentration), detached, and counted 48 hours later. Cell proliferation was determined by counting live cells at the starting and ending times. This method was considered more stringent than [^{3}H]thymidine incorporation because resting cells are alive although their DNA synthesis is minimal. Cell division was determined using carboxyfluorescein-succinimidyl-ester acetate–labeled cells as we described (23). Each experiment was repeated at least twice to verify the results. Nonadherent cells were separated from adherent cells by gentle pipetting. Adherent cells were detached with 0.01 mol/L EDTA. The number of generations and the number of cells in each generation were calculated with the Flow-Jo software for Windows (Tree Star, Inc., Ashland, OR; ref. 24). The percent early and late apoptotic cells were determined with the TACS Annexin V-FITC apoptosis detection kit (R&D Systems, Minneapolis, MN).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Inhibition of tumor cell proliferation was determined with the classic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using an MTT cell proliferation assay kit (Molecular Probes, Eugene, OR) and calculated as described (23).

Statistical analysis. Differences in proliferation, division, and expansion of Gli-1 were calculated using the unpaired Student's t test with the program Statview for Windows.

[6] K. Xie, personal communication 2006.

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Table 1. Design of synthetic miRNAs targeting 3′-UTR Gli-1 mRNA

Gli-1	mRNA sequence (5′→3′)	Free energy (kcal/mol), antisense /sense	Design
3414 sense	CATCCATCACAGATCGAT-3′		
3414 antisense	AUGCGAUCU GU GAUGGAUG	−30.9	3-nt loop in position 10
3414 miRNA	AUGCGAUCUAAAGAUGGAUG	−21.5 (−9.4)	
3418 sense	CATCACAGATCGCATTTCC		
3418 antisense	GGAAAUGCG AU CUGUGAUG	−26.4	3-nt loop in position 10
3418 miRNA	GGAAAUGCGUCACUGUGAUG	−18.9 (−7.5)	
3548 antisense	CAUUAUCAAA UUUCUCCUC		1: Selected the 3′-UTR region with best homology with miR-361
miR-361*	UUAUCA A AUC- UCCAGGGGUAC G		
3548 sense	GAGGAGAAAUUUGAU AAUG		2: The sense strand contains a conserved miR-mer.
miR-136**	ACUCCAUUUGUUUUGAUGAUGGA		
Conserved 8-mer	UUUGAUAA		
3548 antisense	CAUUAUCAA A U UUCUCCUC	−21.9	3: Loop introduction in the antisense strand.
3548 microRNA	5′-CAUUAUCAAUCCUUCUCCUC-3′	−16.0 (−5.9)	
3548 miRNA	3′-GUAAUAGUUAGGAAGAGGAG-5′	−16.0 (−5.9)	
3548 duplex miRNA	5′-CAU UAUCAAUCCUUCUCCUC-3′	−27.9 (+6.0)	4: Complementary sequence with miRNA-3548 for the loop in the sense strand.

*The sequences of miR-361 and miR-136 are reported in refs. 27 and 28, respectively. Calculations of the free-energy of association were made as described in ref. 26.

Results

Design of synthetic Gli-1 miRNA. We analyzed the 3′-untranslated region (UTR) sequence of the Gli-1 and Gli-2 mRNA after the stop codon [nucleotide (nt) 3339] for sequences more likely to form siRNAs containing >30% GC pairs. Two such regions, nt 3414-3438 and nt 3548-3570 (sense), were identified in Gli-1 (Table 1). Similar results were obtained for Gli-2 (data not shown). To design miRNAs from candidate siRNA, we first determined the free energy of interaction (ΔG, kcal/mol) of the sense and antisense strands using the Mfold server (25). Loop-forming nucleotides were introduced in the central position (in 10 of each antisense strand, counting from its 5′ end). We avoided formation of G:C wobbles. The affinity of the loop-containing antisense strand for the sense strand was determined for all possible combinations of the noncomplementary

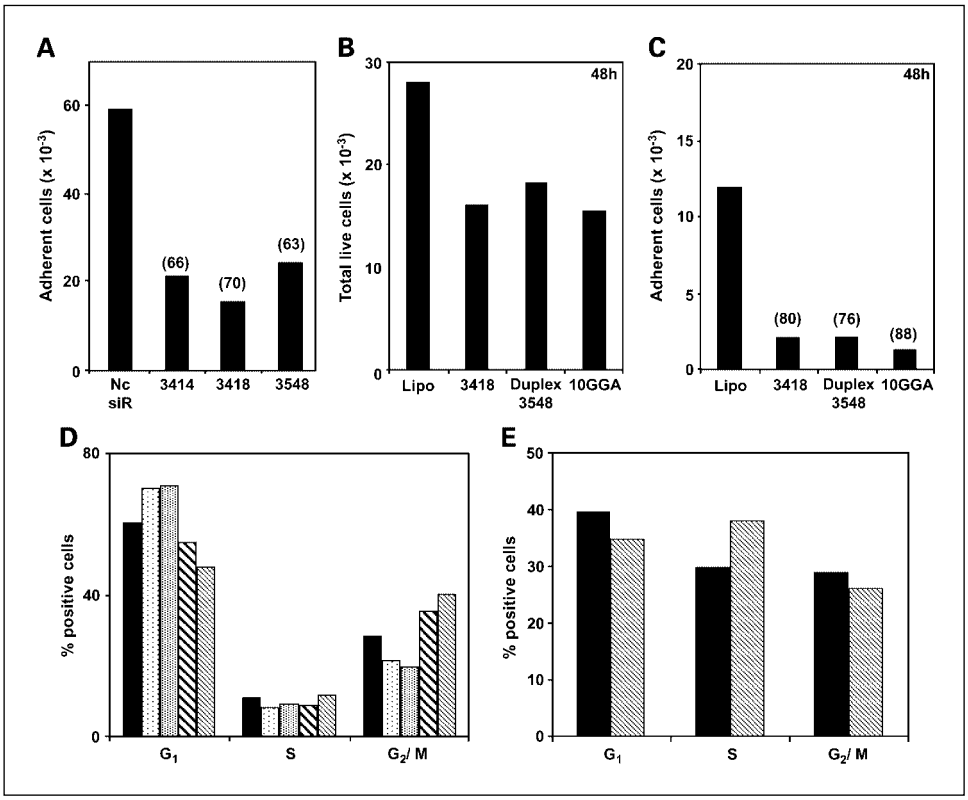

Fig. 1. Synthetic Gli-1-miRNAs inhibited adhesion of tumor cells. *A,* SK-OV-3 cells. Numbers in parentheses indicate the percent decrease in live adherent cells from cultures transfected with miRNAs compared with cultures transfected with negative control siRNA (*Nc siR*) or mock-transfected cells (Lipofectamine 2000). *B* and *C,* MiaPaCa-2 cells. Live cells were counted 48 hours after transfection of 150 nmol/L miRNA. Transfection efficiency ranged between 85% and 93% as determined with negative control siRNA-FITC. *D,* cell cycle analysis in MiaPaCa-2 cell indicates a decrease in cells in G_1 and an increase in cells in G_2-M after interaction with Duplex-3548. ■, HER-2-miRNA-4350-14U; □, HER-2-miRNA-4350-10GGA; ▨, Gli-1-miRNA-3418; ▧, Gli-1-miRNA-3548; ▨, Gli-1 Duplex-3548. *E*, increase in cells in S phase after interaction with Duplex-3548. ■, negative control siRNA; ▨, Gli-1 Duplex-3548.

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nucleotide to the G:U pair (Gli-1 sense, nt 3423-3424) and the A:U pair (Gli-1 sense, nt 3423-3428). We selected mutated antisense sequences that showed the lowest decrease in affinity for the sense sequence compared with that of the nonmutated antisense strand. To identify the functional position of the loop, we constructed two miRNAs, 3414 and 3418, of overlapping sequence. MiRNA-3414 and miRNA-3418 differed in 4 nt. They both contained the 3-nt loops in the positions 10-11.

In addition, we constructed miRNA-3548 and its corresponding duplex miRNA-3548 (Duplex-3548). The decrease in the affinity of association of the loop-forming antisense strand for the sense strand was less than the decrease associated with miRNA 3414-3418. This indicated that the relative affinity of miRNA-3548 for the sense strand was higher than the relative affinity of miRNA 3414-3418 for the sense strand. The affinity of association of the Duplex-3548 strands with each other was higher than the affinity of the antisense strands of 3548 (Table 1). Therefore, it is less likely that the Duplex-3548 strands will dissociate before interacting with mRNA Gli-1, and if they dissociate, both antisense and sense strands will interact with the mRNA. All three selected 3′-UTR sequences were specific for Gli-1 and not present in other genes (Supplementary Table S1).

Gli-1 miRNAs inhibited proliferation of ovarian SK-OV-3 and pancreatic MiaPaCa-2 cell lines. Shh signaling is an essential mediator of pancreatic cancer tumorigenesis. We found that Gli-1 is expressed at high levels in most pancreatic cell lines and in ovarian cell lines (Supplementary Table S2). The ovarian cell line SK-OV-3 contained high levels of Gli-1 and comparable low levels of Gli-2 proteins. We expected that in SK-OV-3 and MiaPaCa-2 cells, Gli-2 will compensate less for the inhibition of Gli-1 than in cell lines expressing higher levels of Gli-2. Gli-1 and Gli-2 siRNAs mediated only transient inhibitory effects on Gli-1 and Gli-2 mRNA levels and proliferation of prostate tumor cell lines. These inhibitory effects were no longer detectable 48 hours after transfection (7). To increase the stringency of analysis, SK-OV-3 cells were stimulated with FCS 24 hours after transfection of Gli-1 miRNAs-3414, -3418, and Duplex-3548. Adherent SK-OV-3 cells decreased in number after transfection with miRNAs and Duplex-3548 compared with that in SK-OV-3 cells transfected with negative control siRNA (Fig. 1A). The decrease in adherent SK-OV-3 cells mediated by miRNAs-3414, -3418, and -3548 was similar. These effects paralleled the decrease in Gli-1 protein levels as determined by flow cytometry (data not shown). Stronger effects were observed 24 hours after transfection in MiaPaCa-2 cells not stimulated by FCS. The decrease in adherent cells induced by Gli-1 miRNAs was similar to the decrease in adherent cells induced by HER-2-miRNA-43550-10GGA (Fig. 1B and C). Because MiaPaCa-2 cells proliferated slower than SK-OV-3 cells, the contribution of nontransfected cells was smaller and the inhibition seemed to be higher. Therefore, designed miRNAs inhibited proliferation and adhesion for 48 hours, and the inhibition was not reverted by stimulation with FCS.

Cell cycle analysis indicated that Gli-1-miRNA-3418 and HER-2-miRNA-4350-10GGA increased the number of cells in resting phase (G_1), whereas Gli-1-miRNA-3548 and

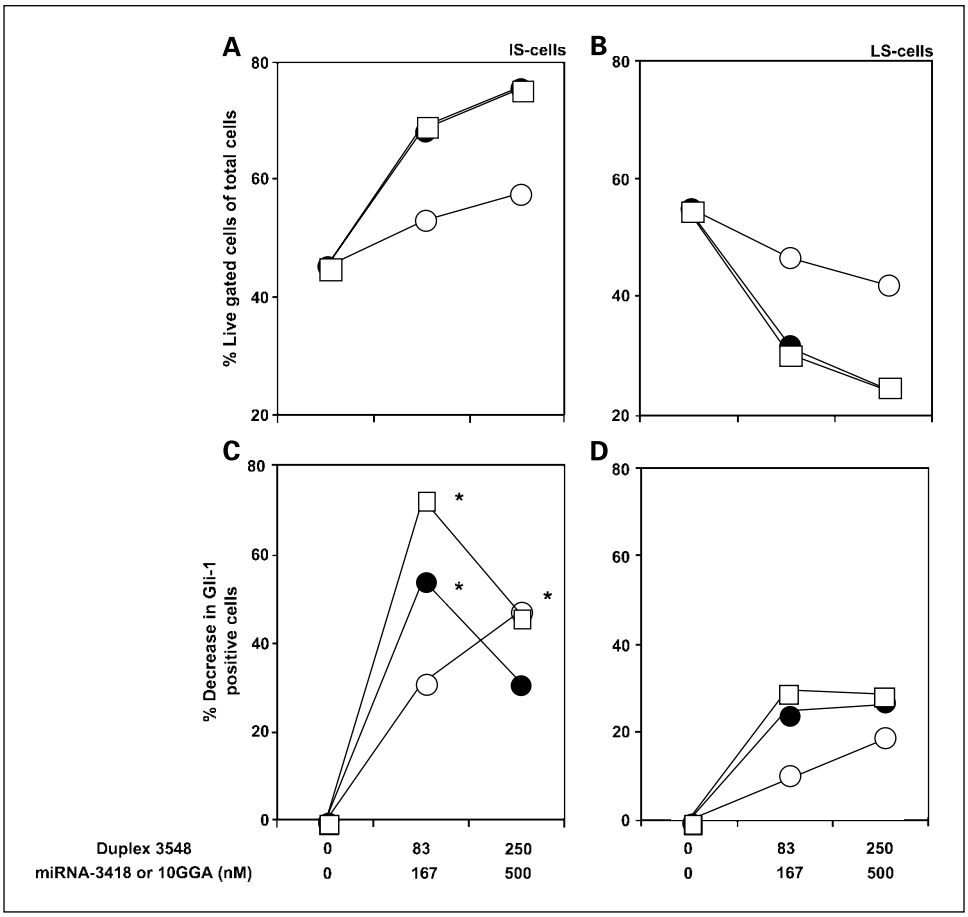

Fig. 2. Gli-1 miRNA decreases expression of Gli-1 protein in tumor cells. *A* and *B,* GLI-1 miRNAs increased the number of intermediate-size (*IS*) tumor cells and decreased the number of large-size (*LS*) cells. *C* and *D,* Gli-1 miRNAs decreased the number of intermediate-size and large-size Gli-1$^+$ cells. ○, Gli-1 Duplex-3548; ●, Gli-1 miRNA-3418; □, HER-2-miRNA-4350-10GGA. *, $P < 0.05$, at least 50% decrease in cell numbers compared with nontransfected miRNAs.

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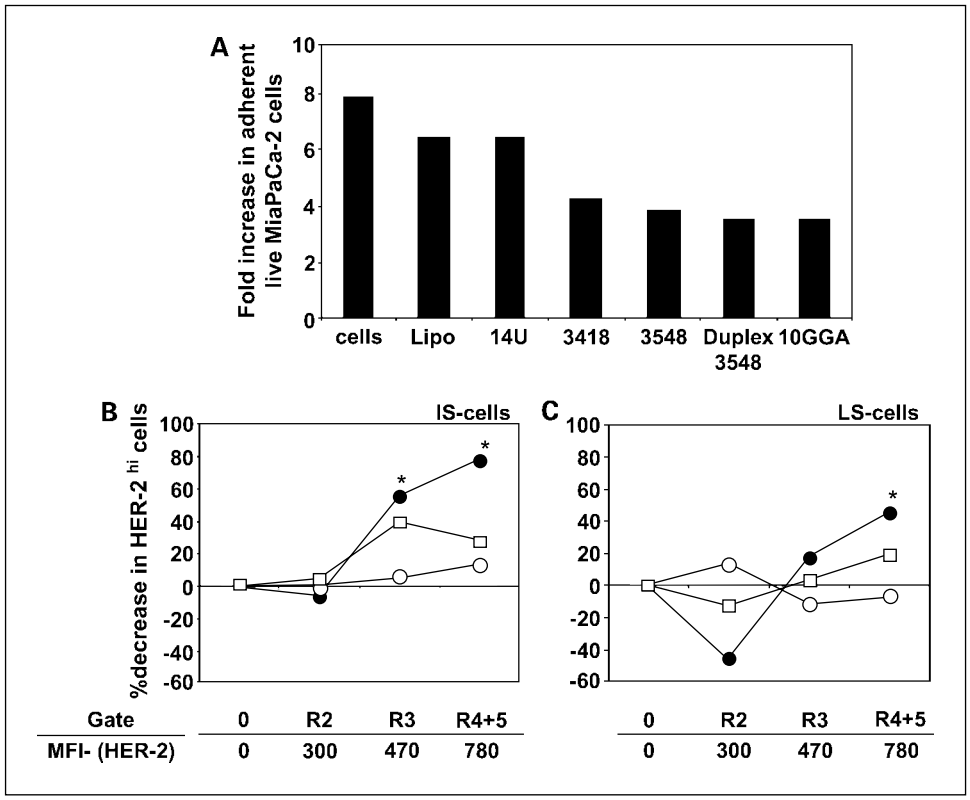

Fig. 3. Duplex-3548 inhibited MiaPaCa-2 proliferation and HER-2 expression. *A,* the fold increase in adherent live MiaPaCa-2 cells was calculated by dividing the number of live cells 48 hours after transfection by the number of MiaPaCa-2 cells initially plated in each well. Cells, untransfected cells; Lipo, mock transfected; 14U, cells transfected with negative control miRNA. *B* and *C,* Duplex-3548 decreased the number of MiaPaCa-2, HER-2^{hi} cells. The percent decrease was calculated in relation to mock-transfected cells. ○, Gli-1 miRNA-3418; ●, Gli-1 Duplex-3548; □, HER-2-miRNA-4350-10GGA. *, $P < 0.05$, at least 50% decrease in HER-2^{hi} cell numbers.

Duplex-3548 decreased the number of cells in resting phase but increased the number of cells in G_2-M phase. This indicated that apoptosis is preceded by G_2-M arrest. At low concentration of FCS (2%), Gli-1-miRNA-3548 had no effect on cell cycle whereas the Duplex-3548 inhibited transition S→G_2-M (Fig. 1D and E). However, Gli-1-miRNA-3548 induced death of more than 50% of MiaPaCa-2 cells.

Gli-1 miRNA decreased Gli-1 protein expression and the size of MiaPaCa-2 cells. Interpretation of results obtained with Western immunoblot and PCR from pooled cells to analyze miRNA effects was complicated by the difficulty of distinguishing between cells in which Gli-1 protein expression was affected and cells in which Gli-1 expression remained unaffected (7). To address whether Gli-1-miRNAs inhibited Gli-1 protein expression, we separately analyzed Gli-1 protein levels in large-size MiaPaCa-2 cells and intermediate-size cells. Smaller-size tumor cells are considered to be metabolically less active than larger-size tumor cells (7). The levels of Gli-1 decreased more in nonadherent cells than in adherent cells (data not shown). miRNAs and Duplex-3548 induced a miRNA concentration–dependent increase in the intermediate-size cells. The intermediate-size MiaPaCa-2 cells doubled in response to Gli-1-miRNA-3418 and HER-2-miRNA-4350-10GGA and increased 1.5 times in response to Duplex-3548. The increase in intermediate-size cells was paralleled by a decrease in large-size cells (Fig. 2A and B). The decrease in the cell size was paralleled by a decrease in the number of Gli-1^+ cells. The decrease in Gli-1^+ cells was higher in the intermediate-size cells (40-50%) and smaller in the large-size cells (<30%). HER-2-miRNA-4350-10GGA also inhibited Gli-1 expression. Therefore, our designed miRNAs significantly decreased the tumor cell size and the expression levels of Gli-1 (Fig. 2C and D). Histograms of Gli-1 and HER-2 expression in each population are shown in Supplementary Fig. S1.

Gli-1 miRNAs inhibited proliferation of dividing MiaPaCa-2 cells and decreased the number of HER-2^{hi} cells. To identify the Gli-1 miRNA that mediated stable inhibition of proliferation, we allowed MiaPaCa-2 cells to divide for 48 hours before transfection. MiaPaCa-2 cells were counted after 48-hour interaction with Gli-1-miRNAs. MiaPaCa-2 cells transfected with Gli-1-miRNA proliferated less than untransfected cells, mock-transfected cells, or cells transfected with negative control miRNA (Fig. 3A). Shh and HER-2 pathways have been proposed to control transition of proliferating tissue stem cells to cancerous stem cells (26). To address whether the Gli-1 miRNAs inhibited HER-2 expression, we determined the decrease in the number of MiaPaCa-2 cells that overexpressed transmembrane HER-2 (designated as HER-2^{hi}). Duplex-3548 decreased the number of HER-2^{hi} cells by 80% and 42%, respectively, in the intermediate-size and large-size MiaPaCa-2 populations (Fig. 3B and C). The decrease induced by the Duplex-3548 was higher than the decrease induced by HER-2-miRNA-4350-10GGA. To identify populations targeted by Gli-1 miRNA, MiaPaCa-2 cells were separated by expression levels of HER-2 into gates (Fig. 3B and C). Intermediate-size MiaPaCa-2 and large-size MiaPaCa-2 cells responded differently to Gli-1-miRNAs. The intermediate-size MiaPaCa-2, HER-2^{hi} cells decreased in number. The decrease was not paralleled by an increase in intermediate-size MiaPaCa-2, HER-2^{lo} cells. In contrast, the decrease in large-size MiaPaCa-2 cells was paralleled by an increase in MiaPaCa-2, HER-2^{lo}. The results suggested that Duplex-3548 eliminated intermediate-size MiaPaCa-2 HER-2^{hi} cells and inhibited HER-2 expression in large-size MiaPaCa-2 cells.

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Duplex-3548 inhibited MiaPaCa-2 cell division. The decrease in numbers of MiaPaCa-2 cells in response to Gli-1 miRNA can result from cell cycle arrest as described above and/or apoptosis of tumor cells. To identify the death pathway, the experiment was repeated under identical conditions with MiaPaCa-2 cells labeled with carboxyfluorescein-succinimidyl-ester acetate. MiaPaCa-2 cells divided at similar rates for the first four divisions in the presence of negative control miRNA or active Gli-1 and HER-2 miRNAs (Fig. 4). A substantially lower number of daughter cells in generations 5 and 6 was found in cultures treated with Gli-1-miRNA-3548, Duplex-3548, and HER-2-4350-10GGA, compared with those treated with Gli-1-miRNA-3418 (Fig. 4C and D). Considering that carboxyfluorescein-succinimidyl-ester acetate–labeled cells divided at least twice in the 48 hours preceding transfection of miRNA, then miRNAs-3548 delayed cell cycle progression.

To address whether Gli-1-miRNA induced death by apoptosis, we determined the numbers of the early and late apoptotic MiaPaCa-2 cells in response to Gli-1-miRNA-3548 and to Duplex-3548. Compared with negative control miRNA, late apoptotic cells increased by 1.78-fold and 2.51-fold, respectively (Fig. 4E-J). HER-2-miRNA-4350-10GGA did not increase the number of late apoptotic cells. We concluded that Gli-1-miRNA-3548, Duplex-3548, and HER-2-miRNA-4350-10GGA inhibited the proliferation of MiaPaCa-2 cells by different effector mechanisms. Gli-1 miRNA and the Duplex mediated

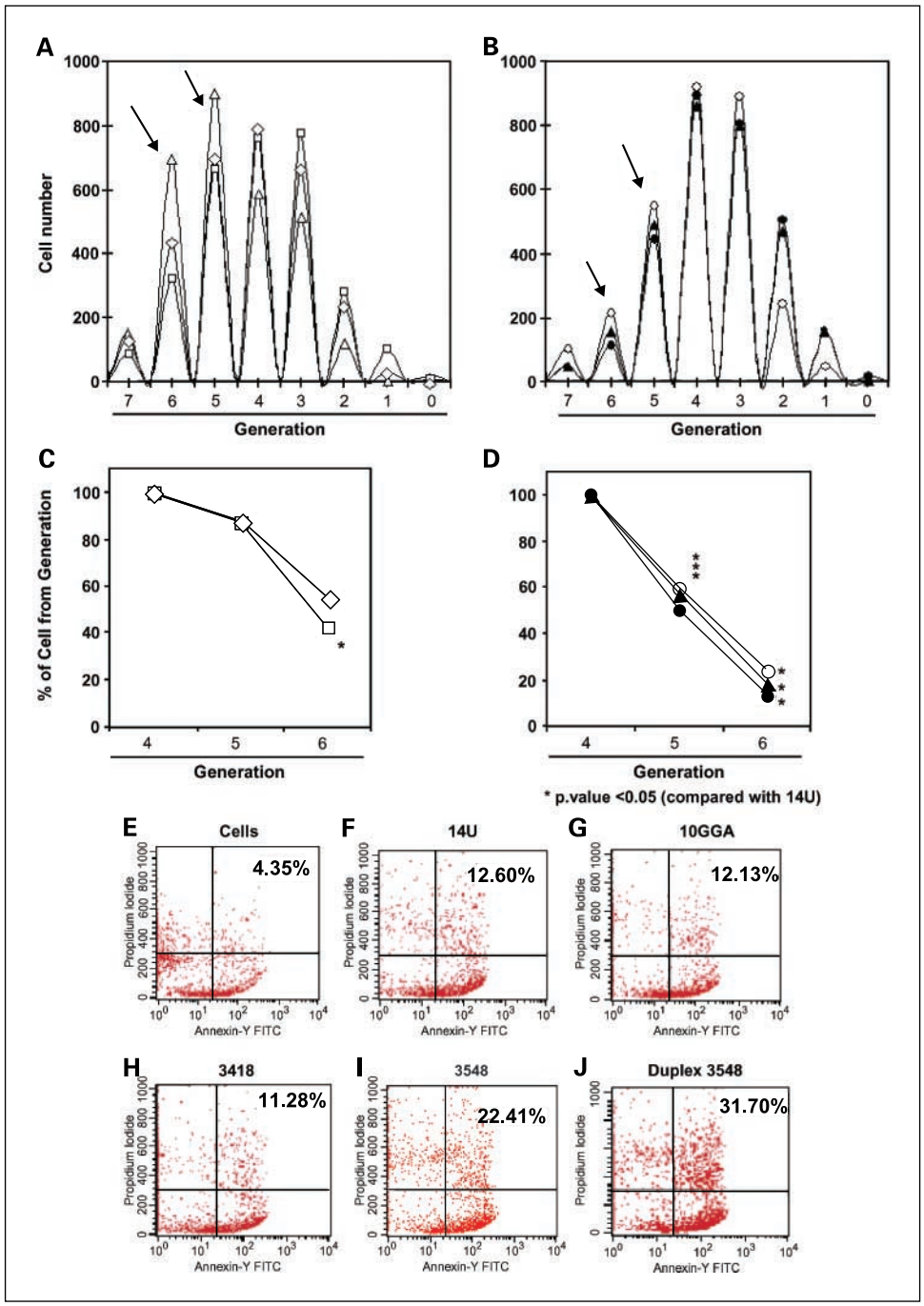

Fig. 4. Gli-1-miRNA-3548 and Duplex-3548 induced late apoptosis. △, Lipo; ■, HER-2-miRNA-4350-14U; ◇, Gli-1 miRNA-3418; ○, Gli-1-miRNA-3548; ●, Gli-1 Duplex-3548; ▲, HER-2-miRNA-4350-10GGA. *A* and *B,* arrows, significant differences in the numbers of daughter cells in generations 5 and 6 in cultures transfected with negative control or Gli-1-miRNA-3548. Equal numbers of cells (n = 3,000) were analyzed. *C* and *D,* the same results as in (*A* and *B*) expressed as percent of generation 4 daughter cells, which was considered 100%. *, $P < 0.05$, at least 2-fold decrease in cell numbers compared with MiaPaCa-2 cells transfected with miRNA-14U and miRNA-3418. *E* to *J,* Gli-1-miRNA-3548 (*3548*) and Duplex-3548 induced late apoptosis. Cells were stained 48 hours after transfection. Cells, control untransfected MiaPaCa-2 cells. Double-positive cells in the top right quadrant [propidium iodide $(red)^+$-Annexin V-FITC $(green)^+$] represent late apoptotic cells. Cells in the bottom right quadrant [propidium iodide $(red)^+$-Annexin V-FITC $(green)^+$ represent early apoptotic cells.

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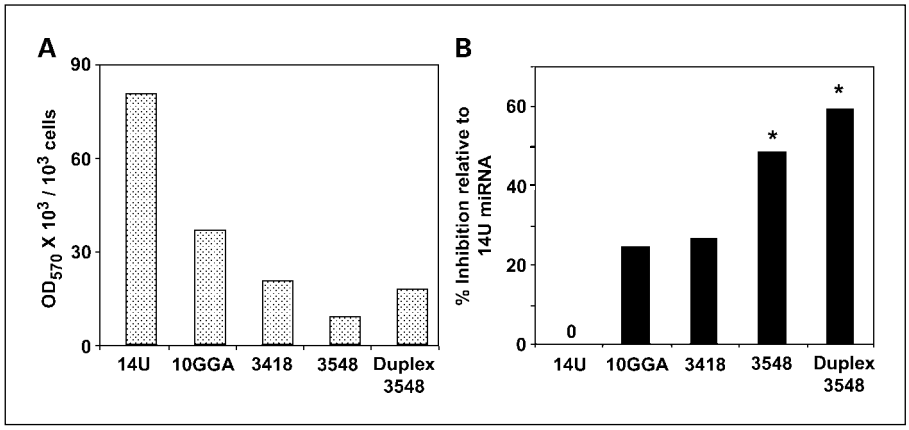

Fig. 5. Inhibited proliferation of MiaPaCa-2 cells surviving miRNAs. *A,* nonadherent cells. *B,* adherent cells. At 48 hours after transfection, equal numbers of cells from each culture were split into five wells and were cultured for an additional 24 hours before MTT assay was done. Results of MTT assay. (Percent inhibition was calculated in relation to the negative control miRNA.) *, $P < 0.05$, at least 50% inhibition compared with MiaPaCa-2 cells transfected with miRNA-14U.

both cell cycle arrest and late apoptosis, whereas HER-2-miRNA-4350-10GGA mediated only cell cycle arrest. This confirmed the result of the previous experiments where miRNA-4350-10GGA increased the number of cells in G_1.

We characterized the proliferation potential of tumor cells that survived Gli-1-miRNA. Results of MTT assay (Fig. 5A and B) confirmed that the inhibition by Duplex-3548 was the most stable. Seventy-two hours after transfection, Duplex-3548 inhibited by >50% the proliferation of surviving adherent tumor cells and almost completely inhibited the proliferation of nonadherent cells. Therefore, designed Gli-1 miRNAs and corresponding Duplex-miRNA can inhibit proliferation of MiaPaCa-2 cells.

Discussion

In this study, we showed the inhibition of proliferation, division, and expression of Gli-1 protein by designed synthetic miRNAs. Synthetic miRNA induced a significant reduction in the numbers of tumor cells in two cell lines that express high levels of the Gli-1 protein.

The stronger inhibitory effects of the Duplex-3548 are probably due to the antisense strand and its sense complementary strand. Each of the strands can interact, while forming a 3-nt loop, with the sense and antisense strands of Gli-1 dsRNA. The Duplex-3548 and miRNA-3548 delayed the division of adherent cells. The delay in division was comparable to the delay induced by a specific miRNA inhibitor of HER-2 translation. However, Gli-1-miRNA and HER-2-miRNA differed in their targeted pathways. Gli-1 miRNA inhibited cell division through accumulation of cells in S and G_2-M and induced late apoptosis of tumor cells. In contrast, HER-2-miRNA inhibited cell division by induction of G_1 arrest but was ineffective at inducing apoptosis. Furthermore, Gli-1 miRNA eliminated HER-2^{hi} cells from a heterogeneous population expressing various levels of HER-2 protein. The causes of the inhibition are unclear.

Duplex-3548 has limited homology with the open reading frame of HER-2 mRNA (Supplementary Table S2). HER-2-miRNA-4350-10GGA has less homology and lower affinity of association with open reading frame of Gli-1 mRNA. Binding by miRNA/duplex miRNA to the HER-2 open reading frame or UTR can be mediated by one of the conserved octamers frequently found in natural miRNAs. Such a conserved octamer is present in the sense strand of Duplex-3548. The antisense strand 3548 has significant sequence homology with the natural miR-361, recently identified in the group of pancreatic islet–specific miRNAs. The Duplex-3548 sense strand is partially homologous with the natural human miR-136 and with several other miRs that have been recently found in embryonic stem cells (26, 27). These findings raise the possibility of natural control of Gli-1 translation by miRs.

Our Gli-1-miRNAs targeted genes associated with cancer metastasis. Inhibiting gene translation offers a therapeutic possibility for cancers resistant to conventional therapies. In a recent study, investigators attempted to inhibit Gli-1 expression in prostate cancer cell lines using siRNA homologous to sequences in the open reading frame of Gli-1-mRNA (7). Those Gli siRNAs had a weak and transient inhibitory effect on proliferation of prostate LNCaP cells containing Gli-2, but significantly inhibited proliferation of DU145 and PC-3 cell lines that lacked Gli-2. The effect could be detected only at 8 and 24 hours after transfection. Inhibition mediated by Duplex-miRNA was observed for 48 hours, and even 72 hours, after transfection.

In conclusion, we designed synthetic miRNAs by introducing 3-nt loops in the middle of candidate siRNAs from the 3′-UTR region of Gli-1 mRNA. A synthetic Duplex-3548 mediated a significant decrease in the number of Gli-1^+ cells and delayed the division of surviving tumor cells. Ongoing studies in our laboratory indicate that Gli-1 miRNA inhibited the motility of MiaPaCa-2 cells.[7] Because Gli-1 controls the pathways of Shh signaling, Gli-1 activation is essential for the progression of pancreatic and small-cell lung carcinoma, and because Gli-1^+ tumors are resistant to chemotherapy, synthetic miRNA directed to Gli-1 may effectively treat pancreatic cancer.

Pancreatic cancer is currently the fourth leading cause of cancer death with very dismal long-term survival rate. Pancreatic cancers are usually diagnosed at advanced stages and they are resistant to conventional chemotherapy and radiation therapy. Therefore, novel approaches are desperately needed for the treatment of pancreatic cancer. That miRNA contains nucleotides of the antisense strand raises the exciting possibility of using negative-strand RNA viruses, such as influenza virus or Newcastle disease virus, for miRNA delivery;

[7] N. Tsuda, unpublished data.

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because such viruses are also oncolytic for some tumor cells, the combination oncolytic virus-miRNA may result in synergistic anticancer effects. The *in vitro* Gli-1 inhibition with miRNA provides a potentially novel approach to treat pancreatic cancer. We are planning to examine the *in vivo* effects of miRNA inhibition in xenograft murine models. It is our hope that miRNA inhibition of target genes (e.g., *Gli-1*) will evolve into clinical trial as a form of treatment for pancreatic cancer.

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