

Lentivirus-mediated antagomir expression for specific inhibition of miRNA function

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ABSTRACT

Micro RNAs (miRNA) regulate gene expression by hybridization and recruitment of multi-protein complexes to complementary mRNA target sequences. miRNA function can transiently be antagonized by antagomirs—chemically modified oligonucleotides complementary to individual miRNAs. Here, we describe the induction of stable loss-of-function phenotypes for specific miRNAs by lentivirus-mediated antagomir expression. Lentivirally expressed antagomirs are transcribed from a H1-promoter located within the lentiviral 3′LTR and were directed against miRNAs encoded on the polycistronic miR17-92 transcript. Functional silencing of miR-18a, miR-19b and miR-20a by the corresponding antagomirs specifically relieves miRNA-mediated reporter gene repression. Inhibition of miRNA function correlates to reduction of ‘miRNA’ amplification by miRNA-specific quantitative RT-PCR. Furthermore, protein expression of E2F-1, a known miR-20 target, is enhanced by lentivirally expressed anti-miR-20 antagomirs in a dose-dependent manner, whereas over-expression of miR-20a reduces E2F-1 levels. Finally, combined over-expression of specific miRNAs and antagomirs reveals individual and complementary functions of miR-18a and miR-20a and demonstrates specific miRNA impact on cell proliferation in a cell culture model.

INTRODUCTION

MicroRNAs (miRNA) are small non-coding RNAs, which are part of an evolutionarily highly conserved intracellular mechanism to regulate gene expression in a sequence-specific manner (1). miRNAs were initially discovered by analysis of mutations causing developmental defects in *Caenorhabditis elegans* (2), but recent work

also demonstrates altered miRNA expression in human cancer including leukemia (3–7). Accordingly, we recently demonstrated BCR-ABL and c-MYC-dependent aberrant expression of the polycistronic miR17-92 miRNA cluster in early phase of chronic myeloid leukemia (CML) (7).

So far, the function of and the targets regulated by individual miRNAs, in particular of those encoded on polycistronic transcripts, are largely unknown. Analysis of miRNA function, for example in the context of clonal evolution, requires methods to generate stable gain- and loss-of-function phenotypes for individual miRNAs and subsequent identification and isolation of modified cells. Stable gain-of-function can be achieved by over-expression of individual miRNAs upon retro- or lentiviral transfer of suitable miRNA-expression cassettes (8–10). In contrast, no method to induce stable loss-of-function phenotypes for individual miRNAs has yet been reported. So far, chemically modified antisense oligonucleotides complementary to specific miRNAs, so-called ‘antagomirs’, have been shown to transiently interfere with miRNA function in cell culture reporter assays and in mice (11–13). However, tracking and isolation of cells with reduced miRNA function has not yet been achieved.

Here we describe the generation of stable gain- and loss-of-function phenotypes for individual miRNAs by lentiviral transfer of antagomir and miRNA expression cassettes, respectively. We demonstrate specific inhibition of miRNA function in reporter assays by lentivirally encoded anti-miRNA ‘antagomirs’ (hereafter referred to as antagomir). This inhibition correlates to reduced ‘miRNA’ amplification by miRNA-specific quantitative RT-PCR (miR-qRT-PCR). Using cell lines transduced with anti-miR-20a antagomirs and sorted according to their EGFP (green fluorescence protein) fluorescence intensity, we demonstrate that protein expression of E2F-1, a known target of miR-20a, correlates inversely with the miR-20a level as determined by miR-qRT-PCR. Finally, combined over-expression of individual miRNAs and antagomirs defines complementary functions of miR-17-92-encoded miRNAs with a negative and positive

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Table 1. Oligonucleotide sequences and predicted transcripts

Name	Sequence (5'–3')
FP-anta-18a RP-anta-18a anta-18a	GATCCC CTATCTGCACTAGATGCACCTTA TTTTGGGAAG TCGACTTCCAAAAAATAAGGTGCATCTAGTGCAGATAGG CUAUCUGCACUAGAUGCACCUAAU
FP-anta-19b RP-anta-19b anta-19b	GATCCC TCAGTTTTGCATGGATTGACACA TTTTGGGAAG TCGACTTCCAAAAAATGTGCAAATCCATGCAAAACTGAGG UCAGUUUUGCAUGGAUUUGCACAU
FP-anta-20a RP-anta-20a anta-20a	GATCCC CTACCTGCACTATAAGCACTTTA TTTTGGGAAG TCGACTTCCAAAAAATAAAGTGCTTATAGTGCAGGTAGGG CUACCUGCACUAUAAGCACUUAAU
FP-control RP-control anta-control	GATCCC AAATCCTTTAGACCGAGCGTGTG TTTTGGGAAG TCGACTTCCAAAAAACACACGCTCGGTCTAAAGGATTGG AAAUCCUUUAGACCGAGCGUGUGUU

impact on cell proliferation of miR-20a and miR-18a, respectively.

MATERIALS AND METHODS

Cloning of H1-antagomir expression cassettes

Self-complementary DNA oligonucleotides (BioSpring, Frankfurt, Germany) encompassing the sequence of the miRNAs miR-18a, miR-19b and miR-20a, as well as an irrelevant sequence (ant-ctrl) were chemically synthesized including overhang sequences from a 5' Bgl II- and a 3' Sal I-restriction site. Annealed oligonucleotides were directionally cloned into the Bgl II/Sal I-digested pBluescript-derived pH1-plasmid as described (14) to generate pH1ant-miR-18a, pH1ant-miR-19b, pH1ant-miR-20a and pH1-ant-ctrl). The oligonucleotide sequences are shown in Table 1. Each sense oligonucleotide harbors a stretch of T as a Pol III transcription termination signal, and the predicted transcript is indicated.

pdC-SEW was used to generate lentiviral transgenic plasmids containing H1-antagomir expression cassettes located in the U3 region of the $\Delta 3'$ long-terminal repeat (LTR) (14). To generate the lentiviral pdCH1-antagomir-SEW plasmid, the pH1ant-mir-18a, pH1ant-mir-19b, pH1ant-mir-20a and pH1-ant-ctrl plasmids were digested with SmaI and HincII and the resulting DNA fragments (~320 nt) were blunt-end ligated into the SnaBI site of pdC-SEW to generate pdCH1-ant-18a-SEW, pdCH1-ant-19b-SEW, pdCH1-ant-20a-SEW and pdCH1-ant-ctrl-SEW, respectively (Figure 1A). All lentiviral vectors encode EGFP as reporter gene driven by a viral SFFV (spleen focus forming virus)-LTR promoter and have an additional regulatory element of Woodchuck hepatitis virus (WPRE) for posttranscriptional processing.

Lentiviral over-expression of miRNAs

The retroviral vector MSCV/LTRmiR30-PIG Δ RI containing the miR-30 backbone was kindly provided by Gerardo Ferbeyre (University of Montreal, Canada). To generate the lentiviral vector S-miR30miRNA-IEW, the plasmid pBluescript was digested with XhoI/HindIII

followed by a DNA polymerase I fill-in reaction and gel purification. The miR-30 cassette was excised from MSCV/LTRmiR30-PIG Δ RI plasmid with AgeI/BglII, and the cohesive ends were filled in using Klenow. The blunt-end ~390 nt fragment was ligated with the blunted XhoI/HindIII vector fragment. Self-complementary DNA oligonucleotides harboring the sequence of the miRNAs miR-18a, miR-19b and miR-20a, were chemically synthesized including overhang sequences from a 5' XhoI- and a 3' EcoRI-restriction site for cloning purposes. Corresponding oligonucleotides were annealed and inserted into the XhoI/EcoRI-digested pBluescript plasmid pmiR30 to generate pmiR30miR-18a, pmiR30miR-19b, pmiR30miR-20a. The correct sequence and insertion was confirmed by DNA sequencing for each plasmid. Finally, these plasmids were digested with SmaI and EcoO109I, and the resulting DNA fragments (~500 nt) were blunt-end ligated into the BamHI site of the bicistronic plasmid SIEW harboring an IRES element (internal ribosome entry site) to generate S-miR30miR-18a-IEW, S-miR30miR-19b-IEW, S-miR30miR-20a-IEW and S-miR30-IEW (control), respectively (Figure 2A). All lentiviral constructs encode EGFP as reporter gene.

Preparation of recombinant lentiviral supernatants and lentiviral transduction

VSV.G-pseudo-typed lentiviral particles were generated by calcium phosphate co-transfection of 293T-cells (maintained in Dulbecco's modified Eagle's medium (DMEM), 10% FCS and 2 mM L-glutamine) and viral supernatants were concentrated as previously described (14). dcH1-antmiR-SEW lentiviral preparations were titrated in triplicate by serial dilutions of the concentrated vector stocks on 1×10^5 K562 cells (maintained RPMI 1640 with 10% FCS) in 24-well plates. The number of EGFP-positive cells were analyzed 72 h post-transduction by FACS analysis (FACS-Calibur, Becton-Dickinson, Heidelberg, Germany). The titers were averaged and typically ranged between $1-5 \times 10^8$ IU/ml.

Lenti- and retroviral supernatants were used to transduce CML-derived K562 cells and to generate stable clones

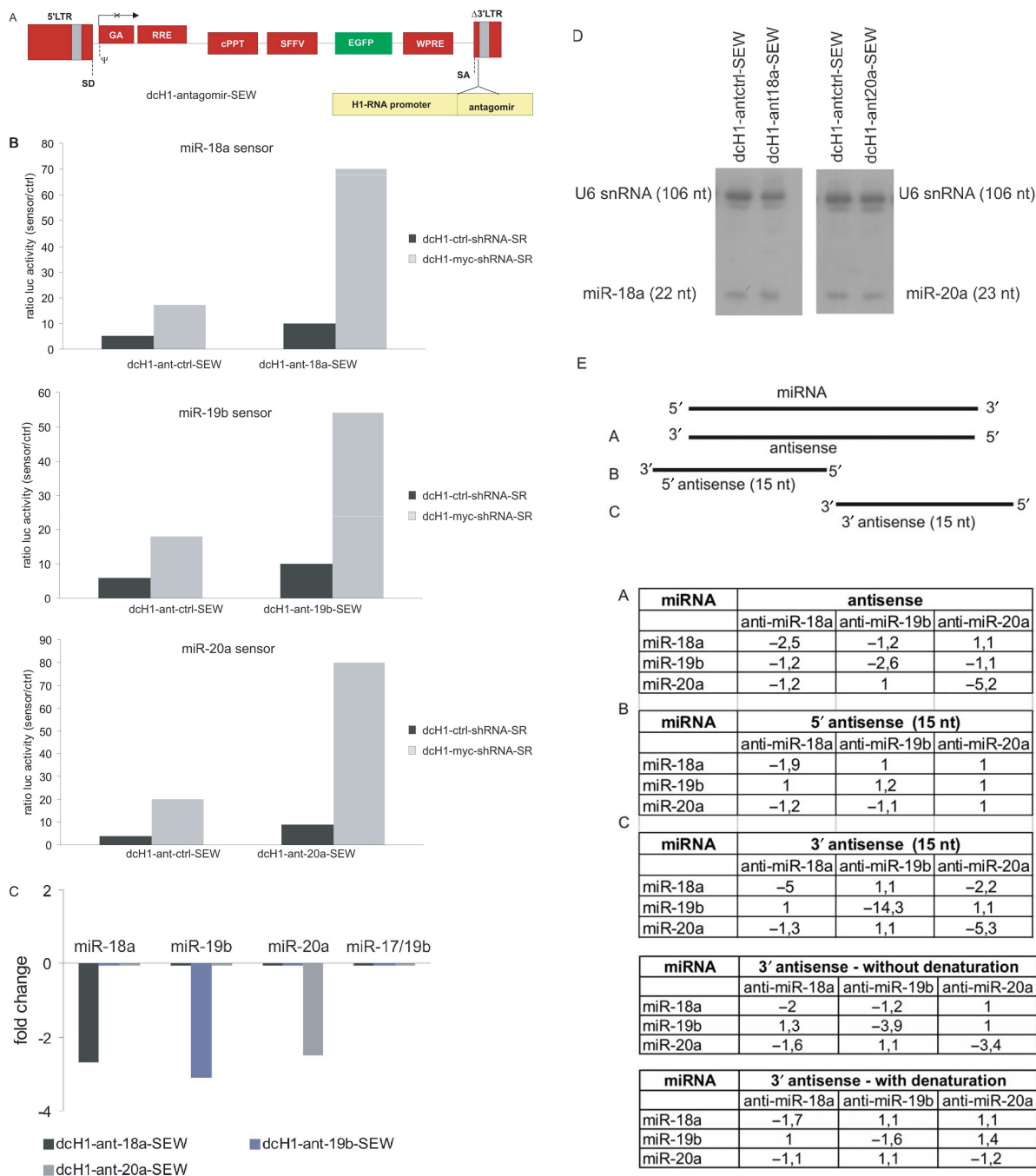


Figure 1. Lentivirus-mediated antagomir expression. (A) Schematic representation of the lentiviral transgene plasmid harboring an antagomir expression cassette with a human H1-RNA promoter inserted into the U3 region of the $\Delta 3'$ LTR. This location results in duplication during reverse transcription indicated as 'double-copy' (dc) vector. S:SFFV-LTR, E:EGFP, W:WPRE (B) Inhibition of miR-18a, miR-19b and miR-20a function by specific antagomirs in luciferase reporter assays. Control- (dark gray bars) or anti-c-myc- (light gray bars) shRNA-expressing K562 cells were stably transfected with miR-18a sensor (upper panel), miR-19b sensor (middle panel) and miR-20a sensor (lower panel) luciferase constructs, respectively. Reporter K562 cells were lentivirally transduced with anti-ctrl, anti-miR-18a, anti-miR-19b and anti-miR-20a antagomirs, respectively, as indicated. The ratio of normalized sensor to control luciferase activity is shown as described (7,16). Mean of two independent experiments are shown. (C) miRNA-specific quantitative RT-PCR (miR-qRT-PCR) for miR-18a, miR-19b and miR-20a, and the polycistronic pri-miR 17-92 transcript in the presence of lentivirally expressed antagomirs anti-miR-18a, anti-miR-19b, anti-miR-20a as compared to control (ctrl)-antagomir. Mean of two independent experiments are shown. miR-16 served as an endogenous control. (D) Northern blot analysis of miRNA expression in the presence or absence of lentivirally expressed antagomirs (miR-18a left panel, miR-20a right panel). U6 snRNA served as a loading control. (E) miR-qRT-PCR of miRNAs in the presence of oligonucleotides complementary to the entire miRNA sequence (A), the 5'- (B) and 3'-half (C), respectively. The table indicates fold changes of miRNA levels as determined by miR-qRT-PCR. (F-H) Cell proliferation of wild-type K562 cells (F), K562 cells over-expressing the miR-17-19b cluster (G), or c-MYC (H), upon lentiviral transduction with anti-miR-18a, anti-miR-19b and anti-miR-20a antagomirs as compared to anti-ctrl transduced cells. K562 cells were lentivirally transduced (transduction efficacy >95%, data not shown), plated at 10^4 cells per well two days after transduction, and the number of trypan blue negative cells was counted. Mean of two independent experiments are shown. The highest cell number on day 14 was set 100%.

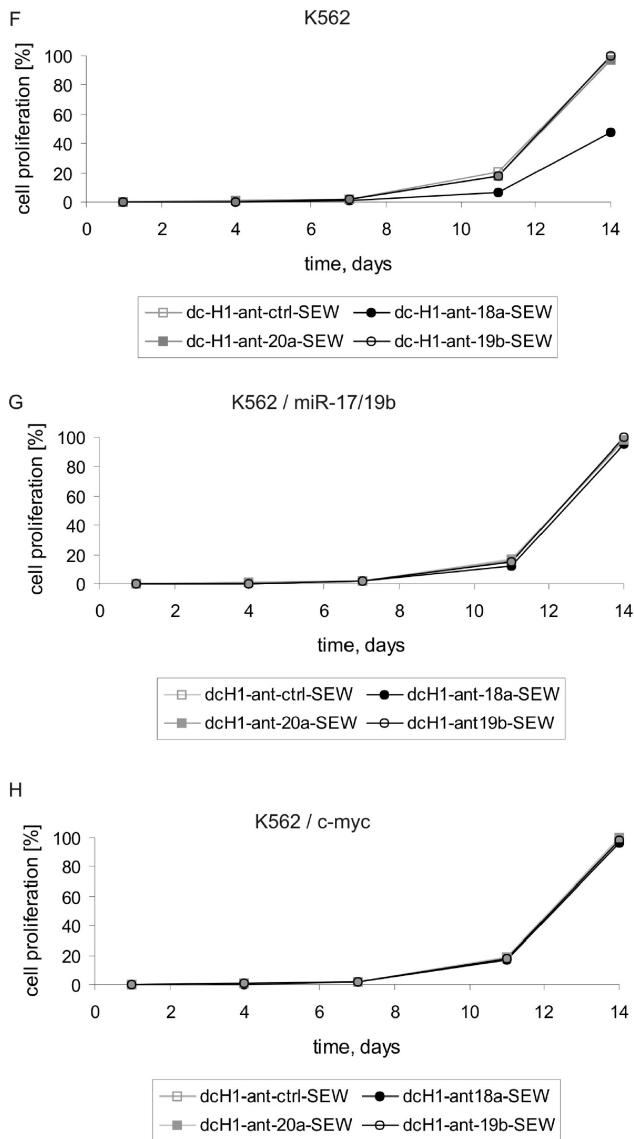


Figure 1. Continued.

of K562/S-miR17/19b-I EW, K562/MSCV-c-myc-IRES-EGFP-WPRE, K562/pGL3-miR-18a-sensor, K562/pGL3-miR-18a-control, K562/pGL3-miR-19b-sensor, K562/pGL3-miR-19b-control and K562/pGL3-miR-20a-sensor, K562/pGL3-miR-20a-control cells each of the latter (K562/pGL3), expressing luciferase as reporter gene as described (7). Lentiviral transduction was performed twice as recently described with an MOI of approximately two (14).

For combined expression of miRNAs and antagonists, K562 cells were first transduced with lentivirus-encoded antagonists. After 72 h (=day 0), miRNAs were over-expressed, and cell counting was performed at the time points indicated in Figure 2E.

Anti-c-myc-shRNAs corresponding to position 817–835 of the human c-myc gene (Gene bank accession no. NM_002467) (7), lentiviral transgene plasmids pdc-SR and shRNA controls were cloned as described (14).

RNA isolation, miR-qRT-PCR and northern blot

Total RNA from K562 cells was prepared using Trizol (Invitrogen). Expression of mature miRNAs was determined by miR-qRT-PCR using miRNA-specific looped RT-primers and TaqMan probes as recommended by the manufacturer (Applied Biosystems, Foster City, USA). Normalization was performed using the $2^{-\Delta\Delta CT}$ method relative to miR-16 miRNA, which was nearly equally expressed in K562 cells in all conditions tested (7, and data not shown). PCR was performed in duplicate using an ABI7500 cyclor.

To study the impact of oligonucleotide annealing on miR-qRT-PCR *in vitro*, we performed an annealing reaction by heating total RNA (10 ng) in the presence of miRNA-complementary oligonucleotides (0.2 μ M/l) for 10 min at 70°C followed by cooling down to 16°C before conventional RT was performed according to the manufacturer. In some experiments shown in Figure 1E, a second heating step for 10 min at 70°C was performed for re-denaturation.

For northern-blotting, total RNA (10 μ g) was separated by electrophoresis using an 16% denaturing polyacrylamide gel and subsequently transferred by electroblotting onto Hybond-N membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization and washing were carried out at 37°C according to standard protocols. Membranes were probed with 32 P-labeled 22–23-nt oligonucleotides corresponding to the respective miRNA, miR-18a and miR-20a and were subsequently visualized by autoradiography. Endogenous U6 snRNA served both as an internal size standard and a loading control (U6sn probe: 5'-TATGGAACGCTTCACGAA TTTGC-3').

Luciferase assay

Luciferase assays were performed 96 h after transduction using the Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to cell numbers for each reaction.

Cell proliferation of transduced K562 cells

A total of 5×10^4 transduced K562 cells/ml were cultured in 24-well plates and viable cells were counted from 1 to 14 days by trypan blue exclusion.

Immunoblotting

Cellular lysates from K562 cells were prepared with lysis buffer (250 mM Tris-HCl, pH7.5, 0.5% Triton X-100; 10 mM NaF; 10 mM Na_3VO_4 ; 1 mM PMSF; 2 mM EDTA; 2 mM AEBSF; 2 μ M Aprotinin; 10 μ M Bestatin; 3 μ M E-64; 4 μ M Leupeptin; 2 μ M PepstatinA) separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Hybond-enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Bioscience, Uppsala, Sweden), and incubated with monoclonal mouse anti-E2F-1 (KH20 + KH95, upstate, biomol, Hamburg) and monoclonal mouse anti- α -tubulin antibodies (DM1A, Calbiochem, Schwalbach am Ts.) according to the manufacturer's protocol. Chemiluminescence was

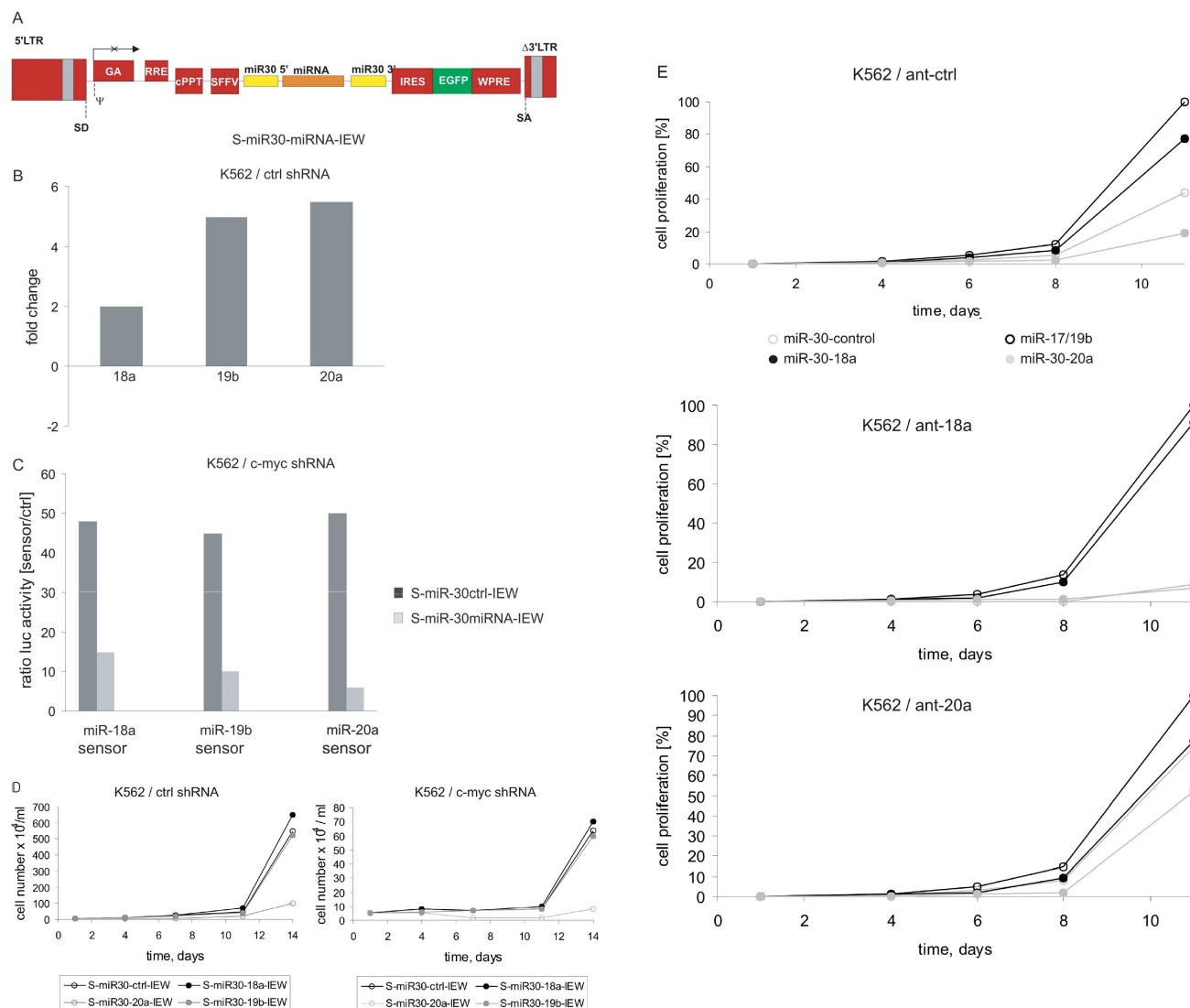


Figure 2. Lentivirus-mediated miRNA-expression. (A) Schematic representation of the lentiviral transgene plasmid harboring a miRNA-expression cassette. Specific miRNA sequences are embedded within sequences derived from miR-30 as described (17). S:SFFV-LTR, I: IRES, E:EGFP, W:WPRE. (B) miRNA expression in control shRNA expressing K562 cells upon lentiviral transduction with miR-18a, miR-19b and miR-20a as determined by miR-qRT-PCR. Mean of two independent experiments are shown. (C) Luciferase activity in K562 cells with reduced c-MYC expression stably transfected with miR-18a sensor (left), miR-19b sensor (middle) and miR-20a sensor (right) luciferase constructs upon lentiviral expression of corresponding control, miR-18a, miR-19b and miR-20a miRNAs, respectively. The ratio of normalized sensor to control luciferase activity is shown as in Figure 1B. Mean of two independent experiments are shown. (D) Proliferation of K562 cells expressing control (left) and anti-c-myc shRNAs (right) upon lentiviral transduction for miRNA expression as indicated. Note the different y-axes in both panels due to impaired cell growth of K562 cells with reduced c-MYC expression (7). (E) Cell proliferation of K562 cells expressing different miRNAs in the presence of lentivirally encoded control antagomirs (upper graph), anti-miR-18a antagomirs (middle graph) and anti-miR-20a antagomirs (lower graph), respectively. Antagomir-transfected K562 cells were transfected with miR-30 control (control), the miR-17-19b cluster (miR17/19b), miR-18a (miR-30-18a), or miR-20a (miR-30-20a), and the number of trypan blue negative cells is indicated as described for Figure 1F–H. Mean of two independent experiments are shown.

used for visualization using the ECL western blotting detection reagents (Amersham Biosciences) according to the manufacturer. Densitometry was done on scanned images using Adobe Photoshop 7.0 software.

RESULTS

Generation of antagomir-encoding lentiviruses

To generate stable miRNA loss-of-function phenotypes, we used our previously described lentiviral vectors, which

encode shRNAs and allow stable transduction and isolation of transduced cells based on lentivirus-mediated EGFP expression (14). In this system, fluorescence intensity of the reporter gene product correlates to the level of shRNA expression and, correspondingly, to the amount of RNAi-mediated inhibition of gene expression. Accordingly, we cloned antagomir-encoding oligonucleotides downstream of a H1-promoter located in the U3 region of the 3'LTR (Figure 1A). During reverse transcription, the H1-promoter-antagomir expression cassette is transferred to the 5'LTR resulting in two

functional antagomir expression cassettes in both proviral LTRs (called dc: double copy vector). In addition, the lentiviral transgenic plasmid drives EGFP expression under control of the SFFV-LTR located within the lentiviral backbone.

We initially analyzed the function of antagomirs against miR-18a, miR-19b and miR-20a which are all encoded on the polycistronic and oncogenic miR-17-92 cluster (15). We used luciferase-based reporter assays with specific miRNA-binding sites in the 3' untranslated region of the luciferase gene using K562 cells as previously described (7,16). In these cells, expression of the miR-17-92 cluster depends on the presence of both the BCR-ABL onco-protein and c-MYC. In addition, transient transfection of chemically modified antagomirs can specifically enhance luciferase activity in these cells (7). To study the function of lentivirally encoded antagomirs, K562 were transduced to more than 95% EGFP-positive cells (data not shown). As shown in Figure 1B, lentiviral transduction with anti-miR-18a, anti-miR-19b and anti-miR-20a antagomirs specifically induces relief of miRNA-dependent luciferase repression, which is further enhanced upon silencing of c-MYC-expression by lentivirus-mediated RNAi. Importantly, this luciferase induction upon lentivirus-mediated antagomir expression corresponds very closely to that observed upon transient transfection of chemical antagomirs as previously described (7). These data demonstrate that lentivirally encoded antagomirs can specifically relieve miRNA-dependent reporter gene repression.

miRNA expression in the presence of lentivirally encoded antagomirs

We next analyzed miRNA expression by miRNA-specific quantitative RT-PCR (miR-qRT-PCR) after transduction with lentivirally encoded antagomirs. miRNA levels are specifically reduced upon lentivirus-mediated antagomir expression whereas the polycistronic pri-miRNA level remains unchanged in transduced K562 cells (Figure 1C). In contrast to miR-qRT-PCR, northern blotting and miCHIP analyses do not detect reduced miR-18a and miR-20a expression under those conditions (Figure 1D and M. Castoldi and M. Muckenthaler, personal communication). Since the latter methods, but not miR-qRT-PCR, include denaturing steps before probe hybridization we asked whether blocking of the 3'-miRNA end may interfere with miR-qRT-PCR. We annealed oligonucleotides complementary to different regions of the miRNA target before reverse transcription as schematically shown in Figure 1E. Hybridization of specific oligonucleotides to the entire (A) and in particular to the 3'-(C) but not the 5'-sequence (B) of individual miRNAs specifically inhibits miR-qRT-PCR for all three miRNAs tested. Furthermore, re-denaturation after addition of oligonucleotides complementary to the 3'miRNA sequence by heating up to 70°C relieves the inhibition of miR-qRT-PCR as shown in both bottom panels (without and with re-denaturation). These data demonstrate that the addition of oligonucleotides complementary to the 3'miRNA sequence can interfere with miR-qRT-PCR *in vitro*, most probably by preventing the

binding of the miRNA-specific looped RT-primer and subsequent inhibition of reverse transcription.

Impact of lentivirally encoded antagomirs on cell proliferation

To study miRNA function on cell proliferation, K562 cells were transduced with lentiviruses encoding specific antagomirs to >95% based on virally encoded EGFP expression (data not shown). Anti-miR-18a, but not anti-miR-19b, anti-miR-20a, or control antagomirs inhibit proliferation of K562 cells (Figure 1F). Interestingly, retroviral over-expression of miR-17-19b, a variant of the miR-17-92 cluster (15) with concomitant elevation of miRNA expression (7) prevents inhibition of cell proliferation by anti-miR-18a specific antagomirs (Figure 1G). Similarly, anti-miR-18a-specific antagomirs have no detectable impact on cell proliferation in K562 cells over-expressing c-MYC (Figure 1H), which induces miR-17-92 expression and enhances proliferation of K562 cells (7). Taken together miRNA-specific antagomirs can specifically modulate proliferation of K562 cells. In addition, enhanced miR-17-92 expression either by retroviral over-expression or by known positive regulators such as c-Myc can overcome the growth inhibitory effects of specific antagomirs.

Combined over-expression of antagomirs and miRNAs

Having demonstrated that lentivirus-mediated antagomir expression can specifically inhibit miRNA function, we cloned lentiviral vectors for expression of individual miRNAs by embedding heterologous miRNA sequences into a miR-30 backbone as previously described (Figure 2A) (17). Increased expression of miR-18a, miR-19b and miR-20a could be demonstrated by miR-qRT-PCR ranging between 2- and about 6-fold (Figure 2B). To study the function of lentivirally expressed miRNAs, luciferase reporter assays were performed similarly as described above. In these experiments, we used K562 cells with reduced c-MYC expression (due to anti c-MYC-RNAi), which have reduced miR17-92 expression (7) and enhanced luciferase activity (Figure 1B). As expected, miRNA-specific repression of luciferase activity is observed for all three miRNAs including miR-18a in these cells (Figure 2C). In addition, over-expression of individual miRNAs in control shRNA transduced K562 and in K562 cells with reduced c-MYC expression reveals enhanced miRNA expression as determined by miR-qRT-PCR (data not shown). In these cells, a specific and strong inhibition of cell proliferation by miR-20a can be detected [Figure 2D, K562 controls (left) and cells with reduced c-MYC expression (right)].

To study the function of specific miRNAs for cell proliferation in more detail, we individually over-expressed miR-30-controls, the polycistronic miR17-19b cluster, miR-18a and miR-20a in K562 cells expressing either control antagomirs (Figure 2E, upper panel), or anti-miR-18a- (Figure 2E, middle panel), or anti-miR-20a antagomirs (Figure 2E, lower panel), respectively. In control-antagomir K562 cells, miR-18a and the polycistronic miR17-19b miRNA increase, whereas miR-20a decreases the number of viable cells as compared to

Table 2. miRNA levels in K562 cells transduced with anti-miR-20a antagonists

	anti-miR-20a	miR30-20a
miR-20a	-4.2	2.2

controls (Figure 2E, upper panel). Expression of anti-miR-18a antagonists in K562 cells inhibits cell proliferation as seen before (Figure 1F, compare controls in Figure 2E, upper and middle panel). However, over-expression of miR-18a and the miR-17-19b polycistron, but not miR-20a miRNAs specifically rescues the growth inhibition mediated by anti-miR-18a antagonists as compared to controls (Figure 2E, middle panel). These data suggest that over-expression of miR-18a and the polycistron functionally out-competes anti-miR-18a antagonists in these cells. Finally, lentiviral expression of anti-miR-20a antagonists can at least partially prevent the growth-inhibitory effect of miR-20a (compare Figure 2E, upper and lower panel). Furthermore, anti-miR-20a antagonists enhance proliferation of control K562 cells similar to that of K562 cells expressing miR-18a (Figure 2E, lower panel). In summary, miRNA-mediated effects on cell proliferation can be identified and antagonized by combined over-expression of miRNAs and corresponding antagonists, respectively.

Dose-dependent increase of E2F-1 protein expression by anti-miR-20a antagonists

E2F-1 is a confirmed cellular target of miR-20a (16). To study the impact of lentivirally encoded antagonists on miRNA-regulated protein expression, K562 cells were transduced with either anti-miR-20a antagonists or miR-20a and the miR-17-19b polycistron, respectively. Expression of antagonists and miR-20a demonstrates a -4.2- and a +2.2-fold induction of miR-20a levels detected by miR-qRT-PCR, respectively, as compared to control cells (Table 2). Whole cell lysates of transduced K562 cells were analyzed for E2F-1 expression by immunoblotting. As shown in Figure 3A and Table 3, expression of both miR-20a (lane 8) and the miR-17-19b polycistron (lane 2) reduce, whereas anti-miR-20a antagonists (lane 5) increase E2F-1 levels.

To analyze dose-response relations between miRNA and target protein expression, K562 cells were lentivirally transduced with anti-miR-20a antagonists and subsequently sorted according to the EGFP-fluorescence intensity. As shown in Figure 3B and Table 5, three populations of transduced cells with different mean fluorescence intensities (MFI) could be isolated. The reduction of miR-20a levels as detected by miR-qRT-PCR (-2.1, -3.3 and -4.0-fold expression as compared to control K562 cells) correlates with the MFI of the respective populations (350, 1250 and 2500). Interestingly, E2F-1 protein expression in these sorted populations increases in parallel with increasing MFI and decreasing miR-20a levels as detected by miR-qRT-PCR (Table 4). These data demonstrate that specific manipulation of

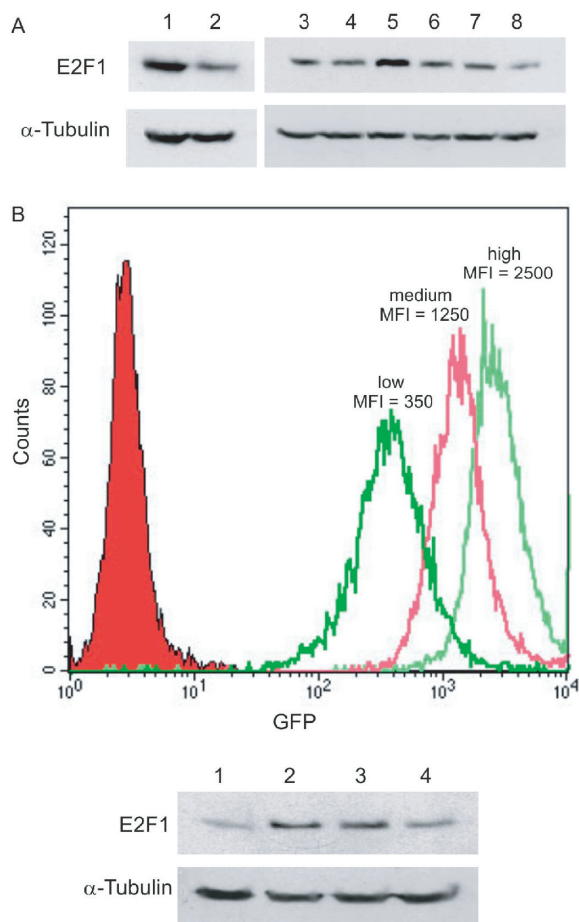


Figure 3. Effects of lentivirally encoded miR-20a and anti-miR-20a antagonist expression on E2F-1 expression. (A) K562 cells transduced with S-17/19b-IEW (lane 2), control dcH1-ctrl-antagonist-SEW (lane 4), dcH1-antagonist-20a-SEW (lane 5), S-mir30-ctrl-IEW (lane 7) and S-mir30-20a-IEW (lane 8), respectively were lysed 4 days after transduction. Lanes 1, 3 and 6 show untransduced K562 cells. The immunoblots were probed with anti-E2F-1 and reprobed with anti-α-tubulin as loading control. (B) Histogram of EGFP fluorescence of untransduced K562 cells (left) and cells sorted according to their EGFP fluorescence after lentiviral transduction with anti-miR-20a antagonists. MFI: mean fluorescence intensity. Below: cellular lysates from the three sorted K562-anti-miR-20a antagonist cell populations shown in Figure 3B were subjected to immunoblotting with anti-E2F1 and anti-α-tubulin antibodies. Lane 1 shows untransduced K562 cells, lane 2, 3 and 4 K562 cells sorted according to high, medium and low green fluorescence intensity, respectively.

miRNA expression can mediate target protein expression in a dose-dependent manner.

DISCUSSION

Our data demonstrate that lentivirus-mediated expression of miRNAs and miRNA-specific antagonists can induce stable gain- and loss-of-function phenotypes for individual miRNAs, respectively. In addition, lentiviral transduction allows identification and purification of transduced cells with modified miRNA expression based on lentivirally encoded reporter gene expression such as EGFP used

Table 3. Densitometric analysis of E2F1 protein expression in K562 cells transduced with anti-miR-20a antagonists

Lane		E2F1/a-tubulin expression [%]
1	K562 'mock'	100
2	K562 'S-17/19b-IEW'	53
3	K562 'mock'	100
4	dcH1-ctrl-antagomir-SEW	93
5	dcH1-antagomir-20a-SEW	199
6	K562 'mock'	100
7	control: S-mir30-ctrl-IEW	85
8	S-mir30-20a-IEW	42

Table 4. miRNA levels in K562 cells transduced with anti-miR-20a antagonists after sorting according to EGFP-fluorescence intensity

miRNA	anti-miR-20a		
	High	Medium	Low
miR-20a	-4	-3.3	-2.1

Table 5. Densitometric analysis of E2F1 protein expression (B) in K562 cells transduced with anti-miR-20a antagonists after sorting according to EGFP-fluorescence intensity

Lane		E2F1/a-tubulin expression [%]
1	K562 'mock'	100
2	dcH1-antagomir-20a-SEW, high	360
3	dcH1-antagomir-20a-SEW, medium	320
4	dcH1-antagomir-20a-SEW, low	168

in this study. Furthermore, EGFP fluorescence of antagomir-transduced cells correlates directly with reduction of miRNA function and concomitant increase of target protein expression in a semi-quantitative manner. Similar dose-response correlations have been observed for stable RNAi after lentivirus-mediated shRNA expression (14).

In our study, antagomir-mediated inhibition of miRNA function was first demonstrated using miRNA-specific reporter assays. In these experiments, antagomirs specifically relieve miRNA-dependent repression of luciferase activity (Figure 1B). In addition, miRNAs levels are specifically reduced in the presence of the corresponding antagomirs if determined by miR-qRT-PCR (Figure 1C). In contrast, northern blot and miCHIP analyses do not detect reduced expression of miR-18a and miR-20a in these cells (Figure 1D and data not shown). Since the latter methods involve a denaturation step before probe hybridization, this difference may be due to hybridization of miRNAs with antagomirs interfering with or preventing primer annealing for reverse transcription. To test this hypothesis in an *in vitro* model, miR-qRT-PCR was performed in the presence of oligonucleotides

complementary to different parts of the miRNA sequences (Figure 1E). We found that hybridization to the 3'- but not 5'-miRNA sequence *in vitro* reduces its detection by miR-qRT-PCR most probably by competition for binding of the looped RT-primer used for this specific reaction. Although the molecular mechanisms *in vivo* mediating reduced miRNA detection by miR-q-RT-PCR in the presence of intracellularly expressed antagomirs are currently not known, the data are in line with a model suggesting that miRNA-antagomir dimers may exist intracellularly for some period of time. Accordingly, miRNA levels detected by miR-qRT-PCR correlate to miRNA function but not to total miRNA levels detectable by northern blotting and/or miCHIP analyses (M. Castoldi and M. Muckenthaler, personal communication). However, the mechanism of miRNA/antagomir duplex formation, their stability and intracellular trafficking, and the kinetic of individual miRNA/antagomir turnover remain to be elucidated.

Target protein expression can be regulated by miRNAs as demonstrated for miR-20a and its experimentally validated target E2F-1 (16). Over-expression of both the miR-17-19b cluster and miR-20a individually decrease whereas lentivirus-mediated anti-miR-20a antagomir expression increases E2F-1 protein expression in K562 cells (Figure 3A and Table 3). The change in E2F-1 protein levels correlates inversely to (free) miR-20a levels as determined by miR-q-RT-PCR and directly to EGFP expression of K562 cells transduced with antagomir-encoding lentiviruses (Figure 3A and B and Tables 4 and 5).

With these tools to modulate miRNA function, we can assign specific functions to individual miRNAs encoded on the polycistronic miR-17-92 miRNA cluster. This cluster is over-expressed in hematological malignancies including early phase CML CD34+ cells (7) and in an erythroleukemia model as recently reported (18). By combined expression of miRNAs and the corresponding antagomirs, we have identified a positive role of miR-18a (and correspondingly a negative impact of anti-18a antagomirs) on proliferation of CML-derived K562 cells. In contrast, over-expression of miR-20a strongly inhibits proliferation of K562 cells, whereas no functional role of miR-19b for cell proliferation has been observed in these experiments. Interestingly, induction of apoptosis upon transient transfection of anti-miR-20a oligonucleotides has recently been described in lung cancer cell lines depending on miR-17-92 expression levels (19). These data may suggest some degree of tissue- or lineage-specificity of miRNA function.

In summary, lentivirus-mediated expression of miRNAs and antagomirs can be used to generate and isolate stable gain- and loss-of-function phenotypes for individual miRNAs in a wide range of target cells. This strategy allows identification of individual miRNA function of polycistronically encoded miRNAs. In addition, it will facilitate the identification of miRNA targets and the sequences involved in regulating target gene expression. However, stable interference with endogenous miRNA expression and function may be accompanied by selection

processes during cell culture similar to that observed for lentivirus-mediated RNAi (20).

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