The **miR-17-92 Cluster of MicroRNAs Confers Tumorigenicity by Inhibiting Oncogene-Induced Senescence**

Lixin Hong, Maoyi Lai, Michelle Chen, Changchuan Xie, Rong Liao, Young Jun Kang, Changchun Xiao, Wen-Yuan Hu, Jiahuai Han, and Peiqing Sun

**Abstract**

In mammalian cells, activation of oncogenes usually triggers innate tumor-suppressing defense mechanisms, including apoptosis and senescence, which are compromised by additional mutations before cancers are developed. The miR-17-92 gene cluster, a polycistron encoding six microRNAs (miRNA), is frequently overexpressed in human cancers and has been shown to promote several aspects of oncogenic transformation, including evasion of apoptosis. In the current study, we show a new role of miR-17-92 in inhibiting oncogenic ras-induced senescence. Further dissection of the miRNA components in this cluster reveals that the miR-17/20a seed family accounts for this antisenescence activity. miR-17 and miR-20a are both necessary and sufficient for conferring resistance to ras-induced senescence by directly targeting p21WAF1, a key effector of senescence. By contrast, these components are not essential for the ability of miR-17-92 to evade Myc-induced apoptosis. Moreover, disruption of senescence by miR-17-92 or its miR-17/20a components leads to enhanced oncogenic transformation by activated ras in primary human cells. Taken together with previous reports that miR-17-92 inhibits apoptosis by suppressing Pten via the miR-19 components, our results indicate that this miRNA cluster promotes tumorigenesis by antagonizing both tumor-suppressing mechanisms, apoptosis, and senescence, through the activities of different miRNA components encoded in this cluster. *Cancer Res* 70(21); OF1–11. ©2010 AACR.

**Introduction**

MicroRNA (miRNA) is a class of evolutionarily conserved noncoding RNAs that suppress the expression of protein-coding genes (1, 2). Recently, miRNA has been implicated in human cancer (3–6). The miR-17-92 cluster is a polycistron encoding six mature miRNAs belonging to four seed families, miR-17 family (miR-17 and miR-20a), miR-18 family (miR-18a), miR-19 family (miR-19a and miR-19b-1), and miR-92 family (miR-92a-1; refs. 2, 7). Overexpression of miR-17-92 has been detected in various types of human cancers (8, 9). When overexpressed, miR-17-92 promotes cell cycle progression and proliferation (10), inhibits apoptosis (11, 12), and induces tumor angiogenesis (13). Furthermore, transgenic mice with moderate overexpression of miR-17-92 in lymphocytes develop a lymphoproliferative disease (14), whereas deletion of this cluster in mouse disrupts normal B-cell development as a result of premature cell death (12). In addition, miR-17-92 accelerates lymphomagenesis from mouse B cells expressing c-Myc by counteracting c-Myc–induced apoptosis (11). Two recent studies have identified miR-19 as the key oncogenic component of miR-17-92, showing that miR-19 inhibits c-Myc–induced apoptosis and promotes c-Myc–mediated lymphomagenesis by repressing the expression of the Pten tumor suppressor gene (15, 16).

In contrast to the antiapoptotic effect of miR-17-92, the role of this cluster in other aspects of tumorigenesis is less well defined. Besides apoptosis, oncogene-induced senescence is another tumor-suppressing defense mechanism that needs to be compromised during cancer development. Instead of tumorigenic phenotypes, activation of certain oncogenes such as ras in early-passage primary cells causes a permanent proliferative arrest known as premature senescence (17–19). Induction of senescence by oncogenic ras is mediated by the p38 mitogen-activated protein kinase (MAPK; refs. 18, 20) and is accompanied by upregulation of inhibitors of cell proliferation, including p16INK4A, p53, p21WAF1, and p14/p19ARF (17, 21, 22). Recent studies show that oncogene-induced senescence occurs in vivo and provides a bona fide barrier to tumorigenesis (23–28).

In the current study, we have shown a new role of miR-17-92 in inhibiting oncogene-induced senescence. Further dissection of miR-17-92 reveals that the miR-17/20a seed family accounts for this antisenescence activity by directly targeting p21WAF1; however, these components are not essential for the antiapoptotic activity of this cluster. Moreover, disruption...
of senescence by miR-17-92 leads to enhanced oncogenic transformation mediated by activated ras. Therefore, miR-17-92 promotes tumorigenesis by antagonizing both tumor-suppressing mechanisms, apoptosis, and oncogene-induced senescence through the activities of different miRNA seed families encoded by this cluster.

Materials and Methods

Cells culture

BJ and WI38 primary human fibroblasts [American Type Culture Collection (ATCC)] were maintained in MEM supplemented with 10% FCS, nonessential amino acids, glutamine, and antibiotics. Grip Tite 293 and 293T (Invitrogen) were grown in DMEM with 10% FCS, sodium pyruvate, glutamine, and antibiotics. These cells were authenticated by the suppliers (ATCC and Invitrogen) based on viability, recovery, growth, morphology, and isoenzymology and had been passaged in our laboratory for fewer than 6 months after resuscitation.

Plasmids

The expression vectors for miRNAs and miRNA clusters were constructed by cloning a single copy or multiple (two or four) copies of their precursor sequences, together with a short upstream and short downstream flanking region, into pLV-EF1α-puro lentiviral expression vectors (Biosettia) or pWAYP. Maps and sequences for these constructs are available on request.

To construct the LacZ-p21WAF1 3′-untranslated region (UTR) reporter, the p21WAF1 3′-UTR sequence was amplified by PCR from the genomic DNA of BJ cells using primers p21-UTR-F (5′-GATCCACCGTCTAGATATACTGCCCAACTAGAGCTC-3′) and p21-UTR-R (5′-TGACATCGAACGGTTACTTTGGAACAGCT-3′) and cloned into pCMV-LacZ (Biosettia).

The short hairpin RNA (shRNA) constructs for p21WAF1, Pten, and Bim were designed based on the single oligonucleotide RNA interference technology (Biosettia). The DNA oligonucleotide for each shRNA was cloned into the lentiviral pLV-EF1α-puro vector, following the manufacturer’s protocol, and verified by DNA sequencing. The sequences of oligos used to generate shRNAs are sh-p21-1, 5′-AAAAGGCTGATCTTCTCCAAACCGAAGATGAGTGGATCACACTCCCTTGGAAGAAGATCAGCC-3′; sh-p21-2, 5′-AAAAGGCTGATCTTCTCCAAACCGAAGATGAGTGGATCACACTCCCTTGGAAGAAGATCAGCC-3′; sh-Pten-1162, 5′-AAAAGGAGGCTTTTGGTTACCTGGTCTATGG-3′; sh-Bim-754, 5′-AAAAGGAGGTATGCAGAGGCTTTTGGTTACCTGGTCTATGG-3′; sh-Bim-655, 5′-AAAAGGAGGCTTTTGGTTACCTGGTCTATGG-3′; sh-Bim-574, 5′-AAAAGGAGGCTTTTGGTTACCTGGTCTATGG-3′; sh-Bim-3, 5′-AAAAGGAGGCTTTTGGTTACCTGGTCTATGG-3′; sh-Bim-2, 5′-AAAAGGAGGCTTTTGGTTACCTGGTCTATGG-3′; sh-Pten-1364, 5′-AAAAGGAGGCTTTTGGTTACCTGGTCTATGG-3′; sh-Pten-1162, 5′-AAAAGGAGGCTTTTGGTTACCTGGTCTATGG-3′; sh-Pten-1162, 5′-AAAAGGAGGCTTTTGGTTACCTGGTCTATGG-3′.

Retrovirus- and lentivirus-based gene transduction

Recombinant retroviruses were packaged and transduced into cells as previously described (29). Recombinant lentiviruses were packaged in 293T cells in the presence of helper plasmids (pMDLg-pR8.9-P and pRSV-REV) using Lipofectamine 2000 (Invitrogen). Cells (1 × 10⁶) were seeded in a 10-cm plate, grown overnight, infected with a multiplicity of infection of 20 in fresh medium containing 8 μg/mL polybrene, and spun for 1 hour at 1,600 to 1,800 rpm. Transduced cells were purified with 120 (BJ) or 50 (WI38) μg/mL of hygromycin B, 600 μg/mL of G418, 12 μg/mL of puromycin, or 5 μg/mL of blasticidin.

Western blot analysis

Western blot analysis was performed with lysates prepared 7 to 10 days after transduction of Ras or MKK3/6 to from subconfluent cells as described (18). Primary antibodies were purchased from Sigma (actin), Santa Cruz (Ras C-20 and p21WAF1 C-19), Cell Signaling (Pten 10386G), and Epitomics (Bim Y36). Signals were detected using enhanced chemiluminescence and captured by the FluorChem 8900 Imaging System (AlphaInnotech).

Analysis of senescence

Senescence in cells was analyzed by measuring the rate of cell proliferation and activity of senescence-associated β-galactosidase (SA-β-gal), as described previously (18). To quantify SA-β-gal–positive cells, at least 200 cells were counted in random fields in each of the duplicated or triplicated wells.

Tumorigenesis assays

BJ were transduced with appropriated oncogenes and miRNAs at PD22. For soft agar colony formation assay, 2 × 10⁵ BJ cells at PD26 were resuspended in a medium containing 0.3% low melting point agarose and plated onto a solidified bottom layer medium containing 0.5% agarose in triplicates in 6-cm plates. Colonies were photographed after 2 to 4 weeks, stained with 0.02% Giemsa, and counted. For tumor formation assays, 2 × 10⁶ BJ cells at PD28 were injected s.c. into the flanks of 4-week-old female HSD/athymic nude mice in 100 μL PBS. Tumor growth was monitored weekly for 10 weeks.

LacZ-p21WAF1 3′-UTR reporter assay

For the LacZ reporter assay, 4 × 10⁵ Grip Tite 293 cells were seeded the day before transfection and transfected with 180 ng of miRNA-expressing vector, 20 ng of LacZ reporter, and 10 ng of a cytomegalovirus-luciferase reporter (Promega) in 96-well plates in triplicates using Lipofectamine 2000 (Invitrogen). Cells were lyzed 24 hours after transfection in 100 μL/well luciferase assay lysis buffer [25 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L EDTA (pH 8.0), 10% glycerol, and 0.1% Triton X-100] and frozen at −80°C for at least 1 hour. After thawing at room temperature, 1 μL of each lysate was mixed with 100 μL of the working solution containing 1.1 mmol/L 3-carboxy-umbelliferyl β-D-galactopyranoside (Sigma) in 0.1 mol/L sodium phosphate (pH 7.3), 1 mmol/L D-galactopyranoside (SA-β-gal), and 0.1% Triton X-100 and frozen at −80°C for at least 1 hour.
MgCl₂ and 45 mmol/L β-mercaptoethanol in a 96-well black wall plate. After incubation at room temperature in the dark for 30 minutes, 50 μL/well of the stop solution (0.2 mol/L Na₂CO₃) were added. Signals (390/448 nm) were measured. Firefly luciferase activity was measured with Luciferase Assay System (Promega) and used as an internal normalization control.

miRNA expression level assays

Expression level of the mature miRNA was measured by TaqMan miRNA Assays kit (Applied Biosystems) following the manufacturer’s protocol. The RUN48 primers were used as a normalization control.

Myc-induced apoptosis

BJ cells seeded in six-well plates at 2 × 10⁵ per well were transduced first with miR-17-92 or its components or a LacZ control and selected with puromycin and then with human c-Myc or green fluorescent protein (GFP) and selected with blasticidin. Two days postinfection, cells were grown in MEM containing 0.5% of FCS for 2 or 6 days, after which both adherent cells and cells in suspension were harvested, washed with PBS, resuspended, and incubated in PBS containing 20 μg/mL of propidium iodide at room temperature for 15 minutes and analyzed by flow cytometry.

Figure 1. The miR-17-92 and miR-106a-363 miRNA clusters disrupt oncogenic ras-induced senescence. A, the expression level of each miRNA encoded by miR-17-92 in BJ cells transduced with a control (Ctrl) or miR-17-92–expressing (miR-17-92) lentivirus. The signals obtained with miRNA–specific primers were normalized to that with the RUN48 primers. The relative miRNA level was calculated by dividing the miRNA level in miR-17-92–transduced cells by that in the control cells. B, the population doublings of BJ cells transduced with miR-17-92 or vector (Ctrl) and Ha-RasV12 (Ras) or vector (VT) were monitored for 12 d starting on day 5 post ras transduction. C, the percentage of SA-β-gal–positive cells in BJ cell populations described in B on day 12 post ras transduction. D, the expression level (in log₁₀ scale) of each miRNA encoded by miR-106a-92 in BJ cells transduced with a control (Ctrl) or miR-106a-92–expressing (miR-106a-92) lentivirus. The signals obtained with miRNA–specific primers were normalized to that with the RUN48 primers. The relative miRNA level was calculated by dividing the miRNA level in miR-106a-92–transduced cells by that in the control cells. E, the population doublings of BJ cells transduced with miR-106a-92 or vector (Ctrl) and Ha-RasV12 (Ras) or vector (VT) were monitored for 12 d starting on day 5 post ras transduction. F, the percentage of SA-β-gal–positive cells in BJ cell populations described in E on day 12 post ras transduction. A–F, values are mean ± SD for triplicates.
Results

The miR-17-92 cluster disrupts oncogenic ras-induced senescence

The involvement of miR-17-92 in cancer prompted us to explore whether it inhibits oncogene-induced senescence (30). When the miR-17-92 cluster was transduced into BJ primary human fibroblasts via a lentivirus, increased expression levels of all six mature miRNAs were detected, ranging from 2-fold to 11-fold over the endogenous levels (Fig. 1A).

Transduction of an activated ras allele, Ha-rasV12, caused a proliferative arrest (Fig. 1B) and increased expression of senescence markers SA-β-gal (Fig. 1C) and MCL1 (ref. 24; Supplementary Fig. S1) in control BJ cells, indicating senescence induction by ras. In contrast, BJ cell expression of miR-17-92 was resistant to ras-induced proliferative arrest and expression of SA-β-gal and MCL1 (Fig. 1B and C; Supplementary Fig. S1). Thus, the miR-17-92 cluster can disrupt oncogenic ras-induced senescence when ectopically expressed in primary BJ fibroblasts. Increased expression of miR-17-92 also...

Figure 2. The miR-17 and miR-20a components are essential for miR-17-92-mediated resistance to oncogenic ras-induced senescence. A, schematic diagrams of miR-17-92 deletion mutants used in the study. B, the expression level of each miRNA encoded by miR-17-92 in BJ cells transduced with vector (Ctrl) or wild-type or indicated mutants of miR-17-92. The signals obtained with miRNA-specific primers were normalized to that with the RUN48 primers. The relative miRNA level was calculated by dividing the miRNA level in miR-17-92 transduced cells by that in the control cells. C, the population doublings of BJ cells transduced with wild-type or indicated mutants of miR-17-92 or vector (Ctrl) and Ha-RasV12 (Ras) or vector (VT) were monitored for 12 d starting on day 5 post ras transduction. D, the percentage of SA-β-gal–positive cells in the BJ cell population described in C on day 12 post ras transduction. B–D, values are mean ± SD for triplicates.
the mutated, but not the other, miRNAs (Fig. 2B). Deletion miRNA components, with abrogation of the expression of seed family (Fig. 2A). When transduced into BJ cells, these mutants were constructed, each containing deletion of a single components to the antisenescence activity, miR-17-92 mutants with deletion of the other seed families (Δ18a, Δ19a&19b-1, or Δ92a-1) were fully capable of blocking oncogenic ras-induced senescence (Fig. 2C, right and D). Similar observations were made in WI38 cells (Supplementary Fig. S2A and B). These data show that the miR-17/20a seed family is essential for the miR-17-92 cluster to prevent ras-induced senescence, whereas the other seed families are dispensable.

We previously showed that oncogenic ras-induced senescence is mediated by the p38 MAPK and that premature senescence can be induced by constitutively active forms of MKK3 (MKK3E) and MKK6 (MKK6E), the upstream kinases of p38 (18). The miR-17-92 cluster also conferred resistance to MKK3E-induced senescence in BJ cells (Supplementary Fig. S2C and D), suggesting that these miRNAs disrupted senescence by targeting a signaling step downstream of p38.

A requirement of the miRNAs belonging to the same family as miR-17/20a was also observed for the antisenescence activity of the homologous miR-106a-363 cluster. Disruption of ras-induced senescence by miR-106a-92 in the absence of miR-363 (Fig. 1E and F; Supplementary Fig. S3) indicates that miR-363 is dispensable. In addition, deletion of miR-106a and miR-20b, but not miR-18b, miR-19b-2, or miR-92a, abolished miR-106a-92–conferred resistance to senescence (Supplementary Fig. S3), suggesting that miR-106a/20b are the only components essential for the senescence bypass by the miR-106a-363 cluster.

**miR-17 and miR-20a are dispensable for the antiapoptotic activity of the miR-17-92 cluster**

To further dissect the oncogenic function of miR-17-92, we determined the requirement of the miR-17/20a components for the antiapoptotic activity. It has been shown that the miR-19 components mediate the evasion of Myc-induced apoptosis in B lymphocytes and acceleration of Myc-induced lymphomagenesis (15, 16). To avoid the cell type–specific effects, we analyzed the antiapoptotic activity of miR-17-92 in BJ fibroblasts, the same cell type in which its antisenescence activity was dissected. Myc was reported to induce apoptosis in growth factor–deprived fibroblasts (31). We thus examined whether the miR-17-92 cluster also counteracted Myc-induced apoptosis in this setting. Compared with the BJ with a GFP control, cells transduced with the human c-Myc oncogene displayed an increased rate of cell death after 2 to 6 days of serum starvation, as measured by the percentage of cells that failed to exclude propidium iodide (Fig. 3). By contrast, in BJ cells transduced with miR-17-92 or mutant miR-17-92 lacking the miR-17 and miR-20a components, Myc-dependent apoptosis was greatly reduced compared with the cells transduced with a vector control (Fig. 3). Therefore, like in mouse B-lymphocytes, the miR-17-92 cluster disrupts Myc-induced apoptosis in growth factor–deprived primary human fibroblasts, and this antiapoptotic function of miR-17-92 does not absolutely require the miR-17/20a components. This finding, in combination with the previous

**The miR-17/20a components are required for the antisenescence activity of miR-17-92**

The miR-17-92 cluster encodes six miRNAs belonging to four different seed families, each with a unique sequence at positions 2–7. To dissect the contribution of these miRNA components to the antisenescence activity, miR-17-92 mutants were constructed, each containing deletion of a single seed family (Fig. 2A). When transduced into BJ cells, these mutants displayed expected expression patterns for the miRNA components, with abrogation of the expression of the mutated, but not the other, miRNAs (Fig. 2B). Deletion

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**Figure 3.** The miR-17/20a components are not essential for the ability of miR-17-92 to disrupt Myc-induced apoptosis. BJ cells were transduced with miR-17-92, mutant miR-17-92 lacking miR-17/20a (Δ17&20a) or vector (Ctrl), and c-Myc (Myc) or GFP control (GFP) and serum-starved for 2 or 6 d. The percentage of apoptotic cells was determined by flow cytometry after staining with propidium iodide. Values are mean ± SD for triplicates.
reports that miR-17-92 blocks Myc-induced apoptosis through miR-19 in B-cells (15, 16), indicate that the antisenesce- 
cence and antiapoptotic activities of miR-17-92 rely on separate components encoded by this cluster.

miR-17 and miR-20a are sufficient for the disruption of oncogenic ras-induced senescence

We next investigated whether the miR-17/20a family by it- 
al itself was sufficient for overcoming oncogenic ras-induced 

senescence. In BJ cells transduced with miR-17 and miR-20a 

only (Fig. 4A), oncogenic ras failed to induce proliferative arrest (Fig. 4B) or accumulation of SA-β-gal (Fig. 4C), indicat- 
ing miR-17 and miR-20a are sufficient to confer the antisenescence activity of miR-17-92. In contrast, none of 

the other miRNAs in the cluster was sufficient for bypassing senescence, because cells transduced with the Δ17&20a mu- 
tant, which mediated the expression of miR-18a, miR-19a, 

miR-19b-1, and miR-92a-1 at levels similar to the wild-type 

miR-17-92 (Fig. 4A), became senescent on ras activation (Fig. 4B, left and C; Supplementary Fig. S1).

miR-17 and miR-20a share identical seed sequences and 

high overall sequence homology and thus likely repress the 

expression of the same genes and have similar biological 

functions. Indeed, BJ cells transduced with lentiviruses en- 
coding four copies of miR-17 (17×4) or miR-20a (20a×4) were 

resistant to ras-induced senescence (Fig. 4B, right and C; 

Supplementary Fig. S1), indicating that miR-17 or miR-20a 

alone is sufficient to overcome senescence. We further exam- 
ined the dosage effect of miR-17 and miR-20a on senescence. 

Lentiviral vectors that encode either a single copy or two or 

four tandem copies of miR-17 or miR-20a were constructed. 

These constructs resulted in copy number–dependent expres- 
sion of miR-17 and miR-20a when transduced into BJ 

cells (Supplementary Fig. S4A and B). The expression levels 

derived from the constructs containing four copies of the 

miRNAs were comparable with the levels mediated by the 

wild-type miR-17-92 cluster, whereas those from the one 

and two copy vectors were consistently lower. Corresponding 

to the expression levels, only the four copy vectors conferred 

complete resistance to ras-induced senescence as the
miR-17 and miR-20a alone or in combination, but not the other miRNAs in miR-17-92, were also sufficient to block ras-induced senescence in WI38 human lung fibroblasts (Supplementary Fig. S2A and B). In addition, miR-17 and miR-20a prevented MKK3E-induced senescence (Supplementary Fig. S2C and D). These results confirm that the miR-17/20a components mediate the antisenescence function in multiple cell strains by targeting signaling downstream of p38.

Taken together, our results show that miR-17 and miR-20a are both necessary and sufficient for disrupting oncogenic ras-induced senescence and thus are the key components mediating the antisenescence activity of miR-17-92. Whereas this manuscript was in preparation, an independent study was published, in which miR-17 and miR-20a were identified from a screen designed to isolate miRNAs that could compromise oncogenic ras-induced senescence in human mammary epithelial cells (32). This finding reinforces our conclusion that the antisenescence effect of the miR-17-92 cluster is attributed to the miR-17/20a components.

miR-17 and miR-20a overcome ras-induced senescence at least partly by inhibiting the induction of p21WAF1 expression

To determine the mechanism underlying the antisenescence activity of miR-17-92, we analyzed the relevance of the known miR-17-92 targets in oncogene-induced senescence. miR-17-92 represses the expression of multiple direct targets, including the E2F transcription factors, Pten, Bim, and p21WAF1 (12, 14, 33–38). Among these targets, the suppression of Pten has been specifically attributed to the miR-19 components (15, 16) whereas that of E2F has been attributed to miR-17 and miR-20a (34). In addition, it has been shown that expression of p21WAF1 can be repressed by the miR-17/20a family (32, 37, 38), although it is unclear whether the other components of miR-17-92 also contribute to p21WAF1 suppression.

Because miR-17 and miR-20a are the key mediators of the antisenescence activity of miR-17-92, we focused on the targets of this miRNA family. Inactivation of E2F promotes, rather than blocks, oncogene-induced senescence (22). Therefore, we reason that suppression of p21WAF1, an important senescence mediator (19), by miR-17/20a may contribute to the antisenescence activity of the miR-17-92 cluster. Indeed, oncogenic ras induced p21WAF1 expression, and this induction was greatly reduced in cells expressing the miR-17-92 cluster (miR-17 and miR-20a), miR-17 alone, or miR-20a alone (Fig. 5A). The ability of miR-17-92 to disrupt p21WAF1 induction depended on the miR-17/20a components, as miR-17-92 containing mutations in miR-17 and miR-20a (Δ17&20a) failed to reduce p21WAF1 expression in BJ cells with activated ras (Fig. 5A). Therefore, miR-17 and miR-20a are the miR-17-92 components that are both necessary and sufficient for mediating the abrogation of ras-induced p21WAF1 expression.

It has been shown that miRNAs belonging to the miR-17/20a family (miR-106a, miR-106b, miR-20b, and miR-17-5) suppress the expression of p21WAF1 by directly binding to its 3′-UTR, which contains two sites with sequences complementary to the seed region of miR-17/20a (32, 37, 38). To confirm that the miR-17-92 cluster directly target p21WAF1 through miR-17 and miR-20a, we analyzed a LacZ reporter fused to human p21WAF1 3′-UTR (Fig. 5B). The reporter

Figure 5. miR-17-92 directly suppresses the expression of p21WAF1 through the miR-17/20a components. A, Western blot analysis of BJ cells transduced with vector (Ctrl), wild-type miR-17-92, miR-17 and miR-20a (17&20a), a mutant miR-17-92 lacking miR-17 and miR-20a (Δ17&20a), or four tandem copies of miR-17 (17×4) or miR-20a (20a×4), and Ha-RasV12 (Ras) or vector (WH) on day 8 post ras transduction. B, activity of the LacZ reporter containing a 3′-UTR of p21WAF1 in the presence of vector (Ctrl), wild-type miR-17-92, miR-17 and miR-20a (17&20a), a mutant miR-17-92 lacking miR-17 and miR-20a (Δ17&20a), or four tandem copies of miR-17 (17×4) or miR-20a (20a×4). The LacZ activity in each sample was normalized to that of a luciferase internal control reporter. The percentage of suppression was calculated by dividing the LacZ activity in miRNA-transduced cells by that in the control cells. Values are mean ± SD for triplicates.
expression was significantly repressed by miR-17-92, miR-17 and miR-20a, miR-17 alone, or miR-20a alone, but not by the Δ17&20a mutant. Therefore, miR-17 and miR-20a, but not any of the other components, mediate the p21WAF1 inhibitory activity of the miR-17-92 cluster by directly targeting the 3′-UTR of p21WAF1. Consistent with the importance of p21WAF1 in senescence (39, 40), silencing of p21WAF1 expression by shRNA led to disruption of ras-induced senescence (Supplementary Fig. S5). This finding further confirms the contribution of the miR-17-20a–mediated suppression of p21WAF1 in the antisenescence activity of the miR-17-92 cluster.

In contrast to p21WAF1, the other known targets of miR-17-92 showed different expression patterns in response to various miR-17-92 constructs (Fig. 5A). Consistent with previous reports (15, 16), Pten expression was suppressed only by the miR-17-92 constructs containing functional miR-19 (wild-type and Δ17&20a), but not by miR-17 and/or miR-20a. Despite previous findings that Bim and E2F1 were miR-17-92 targets (12, 14, 35), we detected no significant suppression of these proteins by any miR-17-92 constructs in our system, possibly due to different experimental conditions. Thus, Pten, Bim, and E2F1 are not specific targets of miR-17 or miR-20a under these conditions. Furthermore, silencing of Pten or Bim expression by shRNA (Supplementary Fig. S6A) failed to block ras-induced senescence (Supplementary Fig. S6B and C). These data further show that the antisenescence function of the miR-17-92 cluster does not involve other miR-17-92 targets, such as Pten, Bim, and E2F1, but specifically relies on miR-17 and miR-20a, which suppress the induction of p21WAF1 by oncogenic ras.

The miR-17-92 cluster and its miR-17/20a components enhance oncogenic ras-mediated cellular transformation

Oncogene-induced senescence is a tumor-suppressing mechanism that needs to be disrupted during cancer development (23, 41). We thus analyzed the contribution of miR-17-92–mediated senescence bypass to oncogenic transformation. Primary human cells, such as BJ fibroblasts, can be fully transformed by the combination of
and miR-20a are the major components that contribute to case of resistance to oncogene-induced senescence, miR-17 to develop tumors in nude mice (Table 1). Thus, like in the model. As expected, BJ cells transduced with E1A, MDM2, and Ha-RasV12 formed colonies in semisolid soft agar medium (Fig. 6A and B) and developed tumors when injected s.c. into nude mice (Table 1). The combination of E1A, miR-17-92, and Ha-RasV12 also resulted in efficient anchorage-independent growth in soft agar and tumor development in nude mice, whereas E1A, Ha-RasV12, and the vector control for miRNA failed to do so (Fig. 6A–D; Table 1). Therefore, miR-17-92 is able to replace the p53 inhibitory function of MDM2 and promote tumorigenesis in cooperation with E1A and oncogenic ras.

Furthermore, consistent with their ability to overcome ras-induced senescence, miR-17 and miR-20a together (Fig. 6C and D; Table 1) or miR-17 or miR-20a alone (Fig. 6A and B; Table 1) also transformed BJ cells together with E1A and Ha-RasV12. In contrast, cells expressing the Δ17&20a mutant of miR-17-92 in combination with E1A and Ras only formed a relatively low number (Fig. 6D) of small (Fig. 6C) colonies on soft agar compared with the wild-type miR-17-92 and failed to develop tumors in nude mice (Table 1). Thus, like in the case of resistance to oncogene-induced senescence, miR-17 and miR-20a are the major components that contribute to the transforming activity of miR-17-92 in the presence of E1A and activated Ras. In addition, miR-373, a miRNA shown to disrupt ras-induced senescence by targeting LATS2 (43), was unable to support anchorage-independent growth together with E1A and ras, and cells expressing miR-373, E1A, and ras only formed one tumor of the six injections. Thus, miR-373 failed to mediate efficient cell transformation with E1A and ras, suggesting that disruption of senescence is not sufficient to cause oncogenic transformation in this system. Therefore, it is likely that, by directly suppressing p53, miR-17-92 and MDM2 contribute to oncogenic transformation in this model via a senescence-dependent, as well as a senescence-independent, mechanism.

Moreover, miR-17-92 and its components failed to transform BJ cells together with activated Ras or with MDM2 and activated Ras in the absence of E1A (Fig. 6C and D), indicating that the essential role of E1A in this system cannot be replaced by miR-17-92. Therefore, the major oncogenic activity of miR-17-92 and its miR-17/20a components in this cellular transformation model specifically overlaps with that of MDM2, but not that of E1A. By inactivating the p53-p21WAF1 pathway, these miRNAs functionally mimic MDM2 in both senescence resistance and oncogenic transformation.

Table 1. miR-17-92 and its miR-17/20a components cooperate with E1A and activated ras to promote formation of subcutaneous tumors in nude mice

<table>
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<tr>
<th>Cell lines</th>
<th>Tumor frequency</th>
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<tbody>
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<td>E1A+Ras+Ctrl</td>
<td>0/6</td>
</tr>
<tr>
<td>E1A+Ras+miR-17-92</td>
<td>6/6</td>
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<td>E1A+Ras+17&amp;20a</td>
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<td>E1A+Ras+20ax4</td>
<td>6/6</td>
</tr>
<tr>
<td>E1A+Ras+miR-373</td>
<td>1/6</td>
</tr>
<tr>
<td>WN+Ras+Ctrl</td>
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</tr>
<tr>
<td>WN+Ras+miR-17-92</td>
<td>0/6</td>
</tr>
<tr>
<td>WN+Ras+17&amp;20a</td>
<td>0/6</td>
</tr>
<tr>
<td>WN+Ras+Δ17&amp;20a</td>
<td>0/6</td>
</tr>
<tr>
<td>MDM2+Ras+Ctrl</td>
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</tr>
<tr>
<td>MDM2+Ras+miR-17-92</td>
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</tr>
<tr>
<td>MDM2+Ras+Δ17&amp;20a</td>
<td>0/6</td>
</tr>
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</table>

NOTE: BJ cells (2 × 10⁶) transduced with indicated combinations of oncogenes and miRNA were injected s.c. into nude mice. The number of tumors arising/number of injections within 10 wk after injection is shown.

Discussion

The oncogenic miR-17-92 cluster is frequently overexpressed in human cancer (6, 7). By showing the role of miR-17-92 in suppressing oncogene-induced senescence, the current study adds a new aspect to the tumorigenic activity of this pleiotropic miRNA cluster. Based on our findings, miR-17-92 is capable of disrupting both apoptosis and senescence, two important tumor-suppressing defense mechanisms triggered on oncogene activation. Furthermore, the antisenescence activity of miR-17-92 is mediated by the miR-17/20a components, which, in contrast, are dispensable for the antiapoptotic function of this cluster.

The miR-17-92 cluster and its miR-17/20a components confer resistance to oncogene-induced senescence at least partly by directly targeting p21WAF1, a key effector of senescence induction. Although the current study focuses on ras-induced senescence, miR-17-92 may also affect other types of senescence, such as replicative and oxidative stress-induced senescence, that rely on activation of the p53-p21WAF1 pathway. However, our results do not rule out the possible involvement of other direct targets of miR-17/20a, which, when downregulated together with p21WAF1, may synergistically abrogate ras-induced senescence. Nevertheless, at least some of the miR-17-92 targets, including Pten, Bim, and E2F1, are unlikely to contribute to the antisenescence activity. By contrast, previous reports showed that direct suppression of Pten expression by miR-19 mediates the antiapoptotic function of miR-17-92 (15, 16). Therefore, the miR-17-92 cluster achieves its various oncogenic functions through distinct miRNA components that suppress the expression of different direct targets.

Interestingly, although miR-17-92 compromised ras-induced senescence in both BJ and WI38 fibroblasts, the block of senescence seemed to be partial in WI38 cells. It
was reported that the p16INK4A levels is much higher in WI38 than in BJ cells and that high p16INK4A levels prevent efficient disruption of senescence on p53 inactivation (44). Thus, complete abrogation of ras-induced senescence in WI38 cells may require simultaneous inactivation of the p16INK4A and p53-p21WAF1 pathways.

miR-106b-25 and miR-106a-363 are two paralogues of the miR-17-92 cluster in mammals (7). Like miR-17-92, the mir-106a-363 cluster is also oncogenic (45). We show that mir-106a-363 also confers resistance to oncogenic ras-induced senescence through its miR-106a/20b components that belong to the same family as miR-17a/20a from miR-17-92. Therefore, at least two of these paralogous miRNA clusters have antisenescence activity. These findings thus provide a mechanism underlying at least part of the oncogenic activity of miR-106a-363.

Using a cell transformation model, we showed that the miR-17-92 cluster replaced MDM2 and cooperated with E1A and activated ras to transform primary human fibroblasts. Furthermore, the transforming activity of miR-17-92 in this model was fully recapitulated by its miR-17-20a components that disrupt senescence, but not by the other components including miR-19 that mediates resistance to apoptosis. This result indicates that, in the presence of E1A and activated ras, senescence, rather than apoptosis, is the main rate-limiting factor for cellular transformation and that inactivation of the p53-p21WAF1 pathway by either MDM2 or miR-17/20a is sufficient to compromise this barrier.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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