An E2F/miR-20a Autoregulatory Feedback Loop*[S]

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The E2F family of transcription factors is essential in the regulation of the cell cycle and apoptosis. While the activity of E2F1–3 is tightly controlled by the retinoblastoma family of proteins, the expression of these factors is also regulated at the level of transcription, post-translational modifications and protein stability. Recently, a new level of regulation of E2Fs has been identified, where micro-RNAs (miRNAs) from the *mir-17–92* cluster influence the translation of the *E2F1* mRNA. We now report that miR-20a, a member of the *mir-17–92* cluster, modulates the translation of the *E2F2* and *E2F3* mRNAs via binding sites in their 3′-untranslated region. We also found that the endogenous E2F1, E2F2, and E2F3 directly bind the promoter of the *mir-17–92* cluster activating its transcription, suggesting an autoregulatory feedback loop between E2F factors and miRNAs from the *mir-17–92* cluster. Our data also point toward an antiapoptotic role for miR-20a, since overexpression of this miRNA decreased apoptosis in a prostate cancer cell line, while inhibition of miR-20a by an antisense oligonucleotide resulted in increased cell death after doxorubicin treatment. This anti-apoptotic role of miR-20a may explain some of the oncogenic capacities of the *mir-17–92* cluster. Altogether, these results suggest that the autoregulation between E2F1–3 and miR-20a is important for preventing an abnormal accumulation of E2F1–3 and may play a role in the regulation of cellular proliferation and apoptosis.

The proper regulation of cellular proliferation and cell cycle progression is critical for the normal development of organisms and the prevention of cancer. Among the numerous factors involved in these processes, the E2F transcription factors play an essential role (1–3). E2F1, along with E2F2 and E2F3, are activators of cell cycle progression and promote the entry of quiescent cells into S phase (4, 5). During G_1, E2F1–3 are inhibited by their association with members of the retinoblastoma protein family (pRb,[5] p107, and p130) (3). In mid to late G_1, hyperphosphorylation of pRb by the cyclinD/cdk4-cdk6 complexes leads to the release of E2F1–3, which bind to specific E2F-responsive promoters, stimulating the transcription of genes involved in G_1/S progression (6, 7). Most cancer cells contain mutations that deregulate the pRb/E2F pathway, which highlights its importance in the control of cellular proliferation. Deregulation of Rb/E2F control can also result in the activation of E2F1-induced apoptosis. Indeed, inactivation of pRb or overexpression of E2F1 promote apoptosis in several cell lines. E2F3 has also been shown to stimulate apoptosis but in a E2F1-dependent pathway (8). E2F1-responsive sites have been found in the promoters of several caspases as well as in other pro-apoptotic targets of p53 (9, 10). E2F1 is also activated by the DNA damage signaling pathway (ATM/ATR) leading to the activation of both p53-dependent and independent, pro-apoptotic pathways (11, 12). Therefore, E2F1 provides direct coupling of the cell cycle and apoptotic machinery, and it has been suggested that cycling cells are primed for apoptosis by E2F1 should proliferation be perceived as aberrant (9).

Besides the control of their activity by association with pRb, E2F1–3 are also regulated by phosphorylation (13), acetylation (14, 15), and ubiquitin-dependent degradation (16). E2F1–3 also regulate their own transcription through E2F-binding sites within their promoters (17, 18). Recently, a novel mechanism of regulation of E2F1 activity has been identified: micro-RNAs (miRNAs) have been found to be important modulators of *E2F1* mRNA translation (19). miRNAs are small 21–23 nucleotides non-coding RNAs that control the stability and/or translation of specific transcripts through the recruitment of the RNA-inducing silencing complex (reviewed in Ref. 20). Recent evidence suggests that miRNAs can regulate the expression of numerous genes (21) and several studies point to the role of some miRNAs in the development of cancer (reviewed in Ref. 22). Among them, miRNAs from the *mir-17–92* cluster have been shown to have an oncogenic activity when overexpressed with c-*myc* in a mouse model of human B-cell lymphoma (23). Interestingly, this cluster is amplified in large-B cell lymphoma and in other malignant lymphomas (24). Moreover, miRNAs from this cluster are overexpressed in lung cancer cells and in

Supplemental Material can be found at:
http://www.jbc.org/cgi/content/full/M608939200/DC1

colon, pancreas, and prostate tumors (25, 26). Another group has also shown that the *mir-17–92* cluster is directly regulated by MYC, and two miRNAs from this cluster, miR-17a and miR-20a, inhibited the translation of the *E2F1* mRNA (19). Altogether, these results suggest that miRNAs from the *mir-17–92* cluster can act as oncogenic miRNAs or "oncomirs" when overexpressed, possibly by acting on key regulators of the cell cycle and apoptosis, like E2F1.

Here, we show that miR-20a, a member of the *mir-17–92* cluster, regulates not only *E2F1* but also *E2F2* and *E2F3* via binding sites in the 3′-UTR of their respective mRNAs. We also report that E2F1–3 directly bind the promoter of the *mir-17–92* cluster regulating its transcription. While overexpression of miR-20a decreased apoptosis in a prostate cancer cell line, inhibition of miR-20a by an antisense oligonucleotide resulted in increased cell death after doxorubicin treatment, pointing to a potential anti-apoptotic role for miR-20a. Altogether, these results suggest that the autoregulation between E2F1–3 and miR-20a is important for maintaining a balance between E2F activities in cellular proliferation and apoptosis.

EXPERIMENTAL PROCEDURES

Plasmids Constructions—The E2Fs 3′-UTRs were amplified from the genomic DNA of HeLa cells using the following primers: E2F1, 5′-TGCTAGTCTAGAGGATTTCTGACAGGGCTTGG-AGG-3′ (sense) and 5′-CTACTAGCTAGCAGAAGGGAAGT-GGAGAATGGGCAT-3′ (antisense); E2F2, 5′-TGCTAGTCTA-GAGGTTCTAGTAAACGGCAGCTGTG-3′ (sense) and 5′-CTACTAGCTACGGGACTTTAAGACGGGCGTCTGATA-3 (antisense); E2F3, 5′-CTACTATCTAGAGATCTAAGGTTTA-TCAGCCTCTGCA-3′ (sense); 5′-CTACTAGCTAGCTGTAC-CAAGTCCAGTGTGTGTGAG-3′ (antisense). The wild type and mutated 3′-UTRs of *E2F1*, *E2F2*, and *E2F3* were cloned into the XbaI site of the pGL3-control vector (Promega). For the mutagenesis, the sequences complementary to the seed of the miR-20a miRNA in each 3′-UTR (GCACTTT) were replaced by the sequence (CGTGAAA). For the promoter analysis, a fragment of 1 kb from the *miR-17–92* cluster promoter containing four E2F-binding sites was amplified from genomic DNA using the following primers: 5′-CGGCTAGCCAGGCT-CAGGTACTGCAGCTG-3′ (sense) and 5′-CCCTCGAGG-CACACAGGTTTCCCTC-3′ (antisense). The PCR product was cloned between the NheI and XhoI sites of the pGL3-promoter vector (Promega). To overexpress miR-20a, we cloned the miRNA precursor after PCR amplification from human DNA with the following primers: 5′-CCGCTCGAGGTCTATC-TGATGTGACAGCTTCT-3′ (sense) and 5′-CCGGAATTCAG-CTGGAGTTCTACAGCTAGCAG-3′ (antisense). The PCR product was subcloned into the retroviral vectors MLP and TMP (S. Lowe, Cold Spring Harbor, NY).

Luciferase Assays—Twenty-four hours before transfection, HeLa cells were plated at 300,000 cells per well in a 6-well plate. The pGL3-control plasmids containing the wild type or mutated 3′-UTRs were transfected (1.0 μg) with pRL-globin (0.1 μg) using Lipofectamine 2000 (Invitrogen). For the analysis of the *miR-17–92* cluster promoter, the pGL3-promoter plasmid containing a fragment of the promoter was transfected (3.0 μg) into HeLa cells (plated at 350,000 cells in 60 × 15-mm

plates 24 h before transfection) in combination with different concentrations (0.1 μg, 0.5 μg, 1.0 μg, or with 1.0 μg of empty vector) of a pBABE plasmid expressing E2F-ER protein fusion and pRL-globin (0.1 μg) using FuGENE 6 (Roche Applied Science). The total quantity of pBABE plasmid was maintained at 1.0 μg by adding the empty vector to the various concentrations of pBABE-E2F-ER used. The media was changed 6 h after transfection for fresh medium containing 4-hydroxytamoxifen (500 nM). Luciferase activity was measured 24 h later. For the analysis of E2Fs activity, 500 nM of 2′-O-methyl oligoribonucleotide were transfected into HeLa cells (plated 300,000 cells per well 24 h before transfection) in combination with either the pGVB2 plasmid containing the p73 promoter (2.0 μg), the pGL2 Basic plasmid containing the HsORC-1 promoter (2.0 μg) or the pGL3-control plasmid containing the 3′-UTR of E2F1 (1.0 μg), with the pRL-globin plasmid (0.1 μg) using Lipofectamine 2000 (Invitrogen).

2′-O-Methyl antisense oligoribonucleotides (ASO) used in this study were: miR-17-5p antisense, 5′-ACUACCUGCACU-GUAAGCACUUUG-3′; miR-20a antisense, 5′-CUACCUGC-ACUAUAAGCACUUUA-3′; let-7 antisense, 5′-CUCUGAA-CUAUACAACCUACUACCUCAAUUUG-3′; and scramble, 5′-AAAACCUUUUGACCGAGCGUGUU-3′. The 2′-O-Me ASO were synthesized by Integrated DNA Technologies. All the luciferase assays were conducted at least three times in triplicate. For each experiment, luciferase assays were performed 24 h after transfection using dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well.

Northern Blot Analysis—Total RNA was extracted using the miRVana kit total RNA extraction protocol (Ambion). 20 μg of total RNA were separated on 15% denaturing polyacrylamide gels, transferred to a nitrocellulose membrane using a semidry transfer apparatus (Bio-Rad), and hybridized using the Ambion protocol provided in the miRvana kit. The probe, complementary to miR-20 was labeled using T4 PNK (New England Biolabs) and [γ-32P]ATP. Hybridization was performed overnight at room temperature and the film was exposed for 5 days.

Cell Viability Assay—Twenty-four hours before transfection, PC3 cells were plated at 100,000 cells per well in a 6-well plate. The 2′-O-methyl oligoribonucleotides (200 pmol) were transfected using Oligofectamine. 6 h after transfection, the medium was changed and fresh medium containing 1.5 μg/ml doxorubicin was added. Cells were harvest 72 h after transfection and treated with trypan blue (Cambrex) to evaluate the cell death ratio. The experiment was conducted three times in triplicate.

For miR-20a overexpression, either plasmid MLP-miR-20a or empty MLP plasmid were transfected in PC3 cells and selected with puromycin. About 10^5 cells per well in a 12-well plate from each of the selected populations were plated in medium with or without doxorubicin (100 ng/ml). After 3 days, all cells (even floating ones) were harvested and stained with 0.4% trypan blue. Percentage of dead cells (blue$^+$) were counted under microscope. Data represent the average of three experiments.

Cell Growth Assay—Either empty MLP vector or MLP-miR-20a vector were transiently transfected into PC3 cells. 24–36 h post-transfection, GFP$^+$ cells were FACS-sorted (FACS Van-
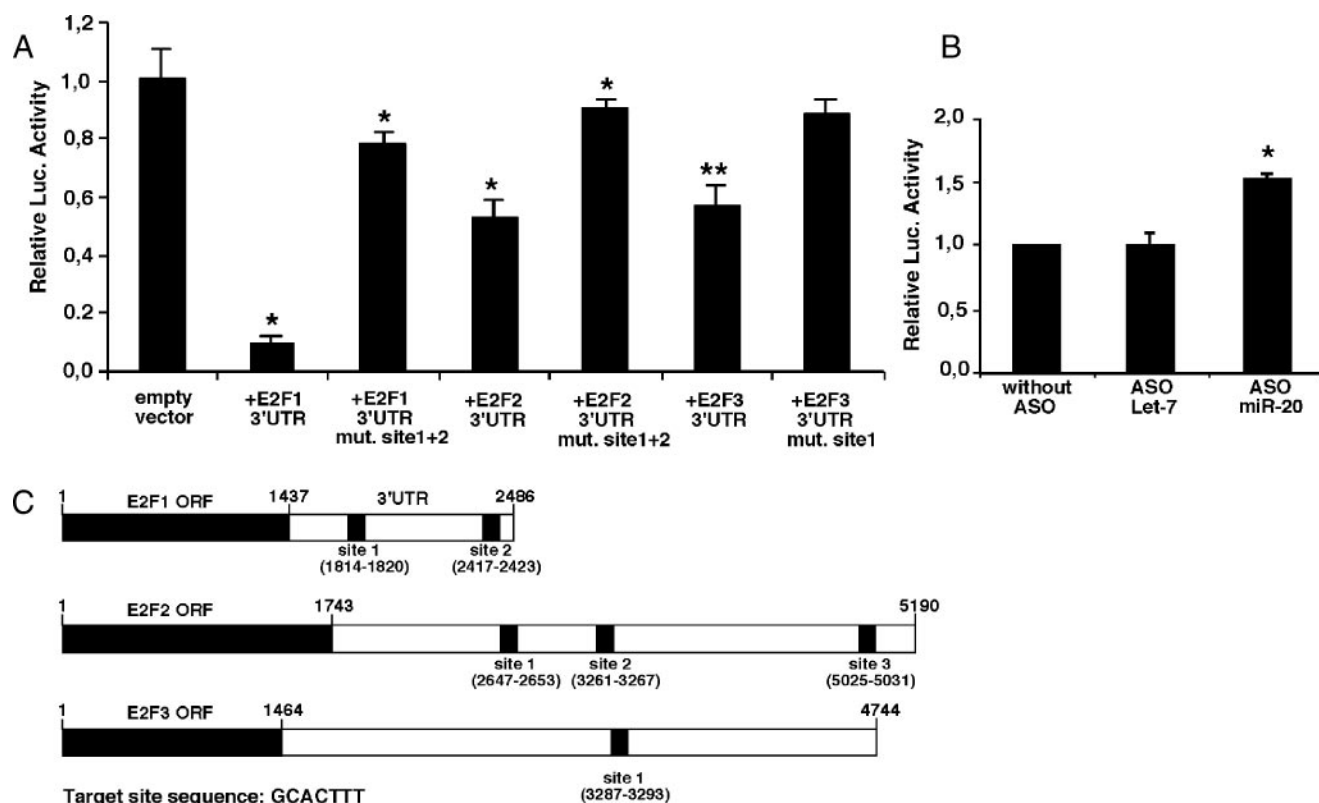
FIGURE 1. **miR-20a regulates E2Fs expression.** *A,* luciferase activity from HeLa cells transfected with a reporter luciferase gene fused to the wild type or mutated *E2F1, E2F2,* or *E2F3* 3′-UTR. *Mut. site,* miR-20a-binding sites are mutated. *B,* luciferase activity from the luciferase-*E2F1* 3′-UTR reporter in HeLa cells transfected with 2′O-Me ASO against miR-20a or the let-7 miRNA as a control. *C,* predicted target sites of miR-20a in the 3′-UTRs of *E2F1, E2F2,* and *E2F3* mRNAs. Paired *t* test: *, $p < 0.01$; **, $p < 0.05$.

tage SE, BD Biosciences, San Jose, CA). 5000 GFP$^+$ cells per well were directly sorted into 24-well plate containing media with or without doxorubicin (125 ng/ml). 72 h post-transfection, media was changed and surviving cells were allowed to grow further for another 3 days. Cells were washed twice with PBS and fixed with 0.5% glutaraldehyde solution in PBS. Cells were then stained with 0.5% crystal violet, rinsed several times with large amount of cold water, and dried. Retained dye, which is proportional to cell mass/number, was extracted with suitable volumes of 10% acetic acid and 100 μl of extracted dye was used for absorbance measurements at A_{595} in 96-wells plate with a microplate reader. Data represent the average of three experiments.

Clonogenic Survival Assay—After transient transfection and FACS sorting (as in the cell growth assay), 500 PC3 GFP$^+$ cells per well were directly sorted into 6-well plate containing media with or without doxorubicin (12.5 ng/ml). 72 h post-transfection, medium was changed, and surviving cells were allowed to form colonies for another 8–10 days. Colonies were fixed with glutaraldehyde, stained with crystal violet, and counted manually. Data represent the average of three experiments.

Western Blot—HeLa cells were trypsinized and washed one time with PBS, and the pellet was resuspended in 500 μl of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 5 min, and the lysates were sonicated 4× for 5 s. The pellet was washed

three times with 500 μl of lysis buffer, one time with PBS, two times with water, and resuspended in 25 μl of 4× Laemli buffer and heated 5 min at 95 °C. The proteins were loaded on a 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The following antibodies were used for Western blot: anti-E2F1 KH-20 mouse (1 μg/ml), anti-E2F2 CC11 mouse (1 μg/ml), anti-α-tubulin (B-5-1-2 1:5000 mouse) (Sigma). Signals were revealed after incubation with anti-mouse secondary antibody (1:1500) coupled to peroxidase (Amersham Biosciences) by using ECL (Amersham Biosciences).

RESULTS

Regulation of E2F1–3 Expression by miR17/miR20 miRNAs— To investigate whether E2F1 expression was regulated by factors binding to its 3′-UTR, we fused the intact 3′-UTR of *E2F1* to a luciferase reporter. We observed a 10-fold reduction in luciferase activity from transfected HeLa cells when compared with a luciferase reporter fused to the 3′-UTR of SV40 (Fig. 1*A*). Since O'Donnell *et al.* (19) reported that the *E2F1* mRNA contained binding sites for miR-17/miR-20 miRNAs that regulated its expression, we mutated the two miR-17-5p/miR-20a-binding sites in the *E2F1* mRNA 3′-UTR. When both sites were mutated, we observed an increase in luciferase activity near to the control level (Fig. 1*A*). Moreover, co-transfection of the luciferase-*E2F1* 3′-UTR reporter with 2′-*O*-methyl ASO against miR-20a resulted in a 50% increase in luciferase activity compared with a let-7 2′-*O*-methyl ASO control (Fig. 1*B*). Together, these data support the previous observation that the
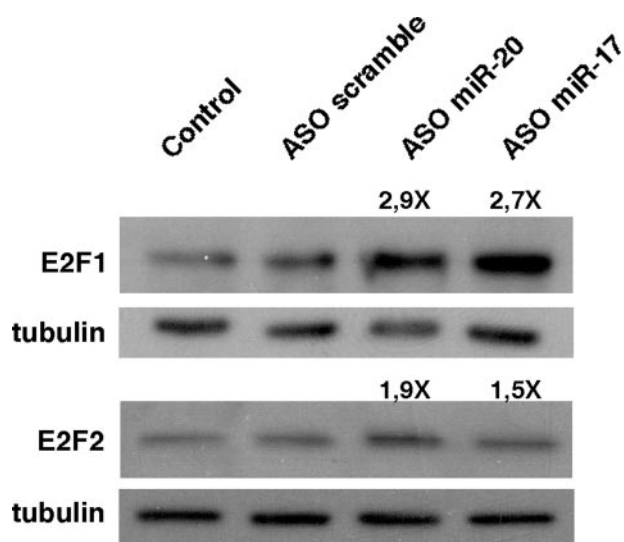
FIGURE 2. **Western blot analysis of endogenous levels of E2F1 and E2F2 in HeLa cells transfected with 2′-O-Me ASO against miR-20a, miR-17, scramble, or without ASO (Control).** Numbers correspond to fold increase compared with control.
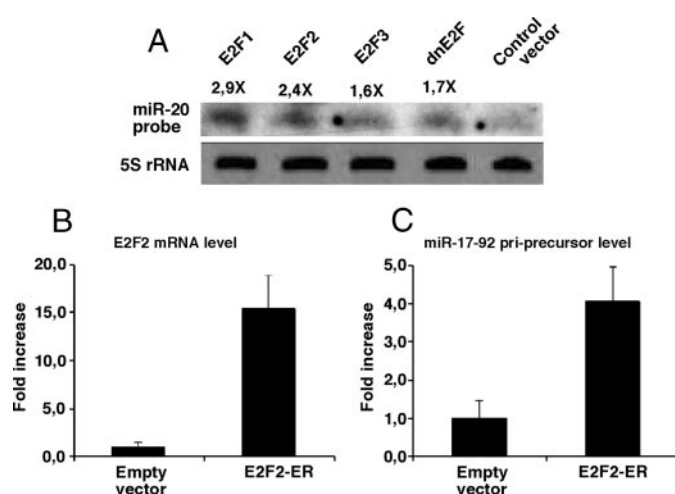

FIGURE 3. **Induction of miR-20a expression by the E2F1–3 transcription factors.** *A*, stable transfection of HeLa cells with pBABE plasmids expressing ER fusions of E2F1, E2F2, E2F3, the defective mutant E2F (*dmE2F*), or the empty vector were induced with tamoxifen for 6 h. Total RNA was extracted and probed by Northern blot for miR-20a expression. The 5 S rRNA (ethidium bromide staining) was used as the loading control. Numbers correspond to fold increase compared with control. *B* and *C*, real-time PCR analysis of the *mir-17–92* pri-precursor. Data are expressed relative to the amount of β-actin mRNA present in each sample. *B*, *E2F2* mRNA. *C*, *mir-17–92* pri-precursor. Details of the real-time PCR protocol are in the supplemental data.

translation of the *E2F1* mRNA is regulated by miRNAs from the *mir-17–92* cluster, including miR-20a, through miRNA-binding sites in the *E2F1* mRNA 3′-UTR (19). Since it is known that other members of the E2F family, especially E2F2 and E2F3, share similar mechanisms of regulation, like Rb binding, acetylation, and co-transcriptional activation (3, 15, 17), we explored the possibility that miR-20a might also regulate the translation of the *E2F2* and *E2F3* mRNAs via binding sites in their 3′-UTR. Indeed, bioinformatics analyses predicted the presence of three miR-20a-binding sites in the 3′-UTR of the *E2F2* mRNA and one site in the 3′-UTR of the *E2F3* mRNA (27) (Fig. 1*C*). Both 3′-UTRs were cloned at the 3′ end of the luciferase gene in the pGL3 control plasmid and transfected in HeLa cells (due to its large size, the *E2F2* 3′-UTR that was cloned contained only the first two miR-20a-binding sites). Compared with the control luciferase reporter, near 2-fold decrease in luciferase activity was observed from the luciferase-*E2F2* 3′-UTR and the luciferase-*E2F3* 3′-UTR reporters (Fig. 1*A*). As we have done for the *E2F1* 3′-UTR, we mutated the binding sites of miR-20a in the luciferase-*E2F2* 3′-UTR and *E2F3* 3′-UTR reporters. As shown in Fig. 1*A*, mutagenesis of the miR-20a target sites increased luciferase activity in both reporter mRNAs near the level of the control, suggesting that binding of miR-20a in these 3′-UTR inhibits luciferase expression. We also determined the consequence of inhibiting miR-20a on the expression levels of endogenous E2Fs. As shown in Fig. 2, transfection of HeLa cells with the 2′-O-Me ASO against miR-20a resulted in an increased level of E2F1 and E2F2, as assessed by Western blot. Unfortunately, even with different antibodies, we have not been able to obtain clean E2F3 Western blots (data not shown). A 2′-O-Me ASO against miR-17, another member of the miR-20a family, also increased both E2F1 and E2F2 levels compared with the control ASO. Altogether, these results suggest that like *E2F1*, the *E2F2* and *E2F3* mRNAs are also targets of miR-20a.

Regulation of mir17/miR-20 Gene Expression by E2F1–3— Since E2F1–3 are transcription factors, we explored the possi-

bility that they may regulate the expression of the miR-20a miRNA. We generated stably transfected HeLa cells expressing tamoxifen-inducible estrogen receptor (ER) ligand-binding domain fusion proteins of E2F1, E2F2, and E2F3 (28). After 6 h of tamoxifen induction, total RNA was extracted from these cells and the level of miR-20a was measured by Northern blot. As shown in Fig. 3*A*, overexpression of E2F1–3 lead to an increased level of miR-20a compared with the control HeLa cells, suggesting that the E2F1, E2F2, and E2F3 transcription factors can induce the expression of miR-20a. We also transfected HeLa cells with a deletion mutant of a E2F-ER fusion (dmE2F), which lack the transactivation domain but retain the DNA-binding domain. Interestingly, induction of this deletion mutant E2F also led to increased miR-20a levels. Other studies have reported such effect of the dmE2F in various cellular systems (2), which suggests that the displacement of a repressive pRb-E2F complex from a promoter by the dmE2F may be sufficient to induce transcription.

The miR-20a miRNA is part of a cluster of seven miRNAs, the *mir-17–92* cluster, which is present on the chromosome 13 (24). This cluster has been shown to be induced by the c-Myc proto-oncogene (19) and amplified in B-cells lymphomas (23). To determine whether the increased expression of miR-20a occurs at the level of the *mir-17–92* cluster precursor, we took advantage of a HeLa cell line stably expressing E2F2-ER. We used real-time PCR to measure the level of pre-miRNA precursor following induction of E2F2. As shown in Fig. 3*C*, induction of the E2F2-ER fusion resulted in an increased precursor levels, suggesting that the *mir-17–92* cluster is up-regulated by E2Fs.

To determine whether this cluster may be directly regulated by the E2F1–3 transcription factors, we looked for putative E2F-binding sites within the first 5kb of the promoter of the *mir-17–92* cluster using SiteScan. E2Fs are known to bind to a canonical TTTSSCGC sequence (where S = C or G) in the
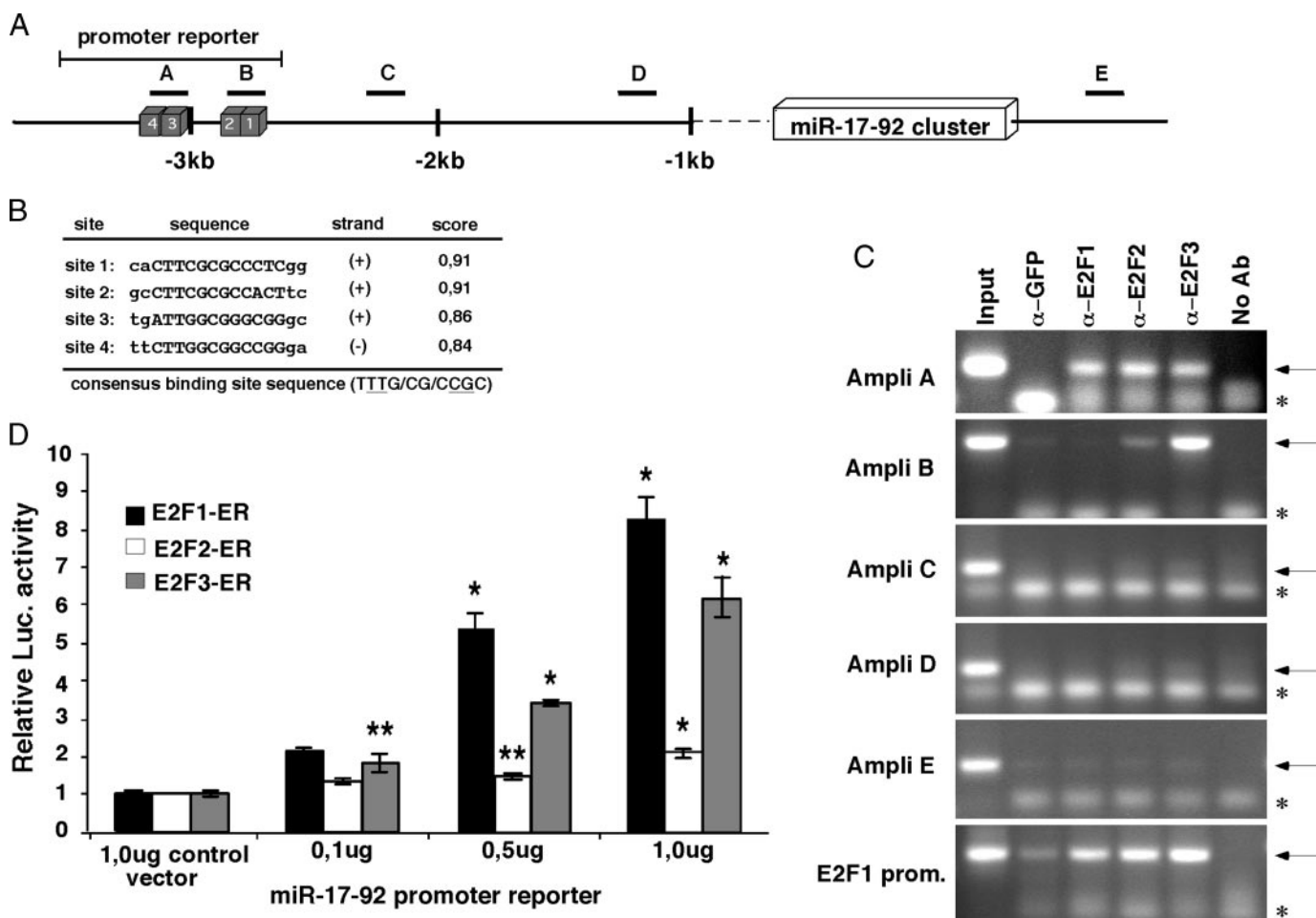
FIGURE 4. **The E2F1–3 transcription factors activate the *miR-17–92* promoter.** *A*, distribution of the putative E2F sites in the promoter of the *miR-17–92* cluster. *Boxes* indicate the four putative E2F sites. Amplicons A to E used in the chromatin immunoprecipitation experiment are indicated by the *horizontal bars*. The 1-kb promoter region cloned is indicated on the figure. *B*, sequences of the predicted E2F-binding sites in the promoter of the *miR-17–92* cluster. *C*, chromatin immunoprecipitation and PCR amplification of fragments from the *miR-17–92* cluster promoter using antibodies against endogenous E2F1, E2F2, and E2F3. Amplicons A to D are from the *miR-17–92* cluster promoter, amplicon E is from the 3′ end of the *miR-17–92* cluster (negative control), and amplicon E2F1 is from the promoter of the *E2F1* gene (positive control). *Input*, PCR amplification from whole chromatin; *GFP*, control antibody against GFP; *No Ab*, PCR reaction without chromatin template. *Arrows* point the PCR products. *Stars* indicate the primers. *D*, luciferase activity from HeLa cells expressing a luciferase gene under the control of a 1-kb fragment of the *miR-17–92* promoter. These HeLa cells were co-transfected with different concentrations (0.1, 0.5, and 1.0 μg) of the pBABE plasmid expressing E2F1, E2F2, and E2F3-ER fusion proteins or the empty vector (1.0 μg). Paired *t* test: *, $p < 0.01$; **, $p < 0.05$. Details of the chromatin immunoprecipitation protocol are in the supplemental data.

promoters of their target genes (29). We identified four putative E2F-binding sites in the *mir-17–92* promoter matching to this sequence (Fig. 4, *A* and *B*). To confirm the binding of these E2Fs to the promoter, we performed chromatin immunoprecipitation experiments to detect the association of the endogenous E2F1, E2F2, and E2F3 transcription factors with the *mir-17–92* promoter in HeLa cells (Fig. 4*C*). In the *mir-17–92* promoter, we generated amplicon A, which is close to two putative E2F-binding sites (sites 3 and 4), amplicon B, which overlaps two other E2F-binding sites (sites 1 and 2), and amplicon D, which overlaps the c-Myc-binding sites identified previously (19). Amplicon E was used as a negative control, while amplicon E2F1 in the *E2F1* promoter was used as a positive control. As shown in Fig. 4*C*, amplicons A and B were amplified after chromatin immunoprecipitation with anti-E2F2 and anti-E2F3 antibodies but not with an anti-GFP antibody. Only the amplicon A was amplified with the anti-E2F1 antibody. Amplicons C and D were negative. Interestingly, a stronger PCR amplification of the amplicon B was observed with E2F3 compared with E2F2 or

E2F1. It may either mean that E2F3 binds more strongly to this region of the promoter or that the E2F3 antibody is more efficient for immunoprecipitation.

To confirm that the *mir-17–92* cluster is regulated by the E2Fs, we cloned a 1-kb fragment of this promoter, containing the four E2F-binding sites overlapping the amplicons A and B (sites 1–4), upstream of the luciferase gene in the pGL3-Promoter plasmid. Co-transfection of this reporter luciferase plasmid with increasing amount of the E2F-ER plasmids resulted in an increased activation of the luciferase gene (Fig. 4*D*). E2F1 showed a stronger activation of this promoter (~8-fold), followed by E2F3 (~6-fold), whereas E2F2 activation was smaller (~2-fold). While E2F2 was as efficient as E2F1 and E2F3 to increase miR-20a levels (see Fig. 3), it may bind to sites that are outside of the 1-kb fragment cloned in the plasmid reporter. Similar levels of induction were observed using an E2F inducible ORC-1 promoter-luciferase reporter (Fig. 1 in the supplemental data). These results indicated that this fragment of the *mir-17–92* cluster promoter is responsive to the E2F1–3 transcription factors.
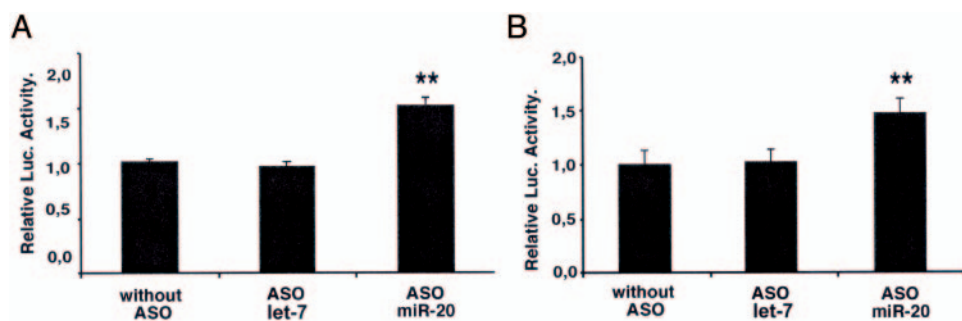
FIGURE 5. *A*, effect of the inhibition of endogenous miR-20a by 2′-*O*-Me ASO on the activity of an E2F-regulated ORC-1 promoter upstream of the luciferase gene. A 2′*O*-Me ASO against let-7 was used as a control. *B*, same experiment as in *A* but with an E2F1-regulated p73 promoter upstream of the luciferase gene. Paired *t* test: **, $p < 0.05$.
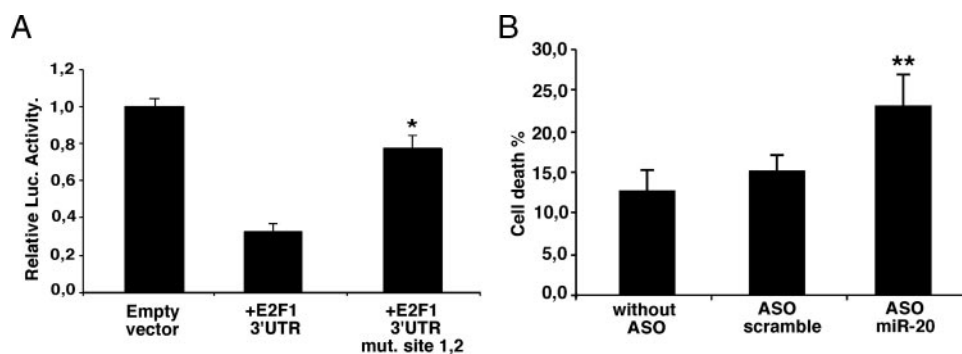

FIGURE 6. **Inhibition of miR-20a increased the susceptibility of PC3 cells to cell death after doxorubicin treatment.** *A*, luciferase activity from PC3 cells transfected with a reporter luciferase gene fused to the wild type or mutated *E2F1* 3′-UTR. *Mut. site*, miR-20a-binding sites are mutated. *B*, PC3 cells were transfected with 2′-*O*-Me ASO against miR-20a or with a scramble ASO. As another control, PC3 cells were transfected without ASO. Cell death was measured by trypan blue staining. Paired *t* test: *, $p < 0.01$; **, $p < 0.05$.

Altogether, these data suggest a self-regulatory mechanism, where the translation of the *E2F1–3* mRNAs is controlled by the miR-20a miRNA, which itself is regulated by the E2Fs at the transcriptional level.

Functional Consequences of the Regulation of E2F1–3 by miR-17/miR-20—As an inhibitor of E2F1–3 expression, miR-20a should block E2F-dependent gene expression and may act as an anti-proliferative and/or as an anti-apoptotic agent. To explore these questions, we first measured the impact of inhibiting the function of miR-20a on the activity of endogenous E2F1–3. To do so, we transfected HeLa cells with plasmids containing luciferase reporter genes under the control of the ORC-1 or p73 promoter, both being E2F-inducible promoters (30, 31). The same cells were co-transfected with a 2′-*O*-Me ASO inhibitor against the endogenous miR-20a miRNA. As shown in Fig. 5, inhibition of miR-20a resulted in a 50% increase in the activation of the ORC-1 promoter (Fig. 5*A*) and p73 promoter (Fig. 5*B*). A control 2′-*O*-Me ASO against the let-7 miRNA did not result in the induction of these promoters. Such induction correlates with the increased E2F1–3 expression observed when HeLa cells were transfected with the miR-20a 2′-*O*-Me ASO (see Fig. 2) and suggests an increased E2F1–3 activity in these cells.

To explore the biological impact of an increased or decreased miR-20a activity, we took advantage of a PC3 prostate cancer cell line that was previously used to study apoptosis after treatment with the DNA damage agent doxorubicin (32). In these cells, an increased E2F activity correlated with apoptosis after treatment with doxorubicin (32). We detected an endogenous miR-20a activity in PC3 cells by measuring a decreased luciferase activity from the luciferase-*E2F1* 3′-UTR wild type compared with the 3′-UTR with mutated miR-20a target sites (Fig. 6*A*). To determine whether the inhibition of miR-20a function increases the level of apoptosis of PC3 cells, they were transfected with the anti-miR-20a or a scrambled 2′-*O*-Me ASOs and treated with doxorubicin. Cell death was measured by trypan blue staining after 3 days in culture. As shown in Fig. 6*B*, transfection of the 2′-*O*-Me ASO against miR-20a resulted in a 50% increase in cell death after doxorubicin treatment compared with the scramble oligonucleotide, suggesting that inhibition of miR-20a makes these cells more sensitive to drug-induced apoptosis. We also overexpressed the miR-20a miRNA in PC3 cells by transfection of a MLP plasmid expressing a miR-20a shRNA. Northern blot from cells transfected with the MLP-miR-20a plasmid revealed an higher expression of the miR-20a miRNA (Fig. 7*A*). Overexpression of miR-20a resulted in a 2-fold decrease in the endogenous level of E2F1 and E2F2, as shown by Western blot (Fig. 7*B*). The growth of these cells was affected by the overexpression of miR-20a, suggesting that it may abolish E2F1–3 expression to an extent sufficient to compromise cell cycle transitions (data not shown). However, after treatment with doxorubicin, PC3 cells overexpressing the miR-20a miRNA showed a 2-fold decrease in cell death compared with the control cells (Fig. 7*C*). By measuring cell survival, after doxorubicin treatment, with a clonogenic survival assay, we observed a 2-fold increase in cell survival of PC3 cells overexpressing miR-20a compared with control cells with the empty vector (Fig. 7*D*). We also measured cell accumulation in monolayers, after doxorubicin treatment, of PC3 cells overexpressing or not miR-20a. Again, when miR-20a was overexpressed, we observed four times more cells in comparison to the control cells (Fig. 7*E*). Both clonogenic and population growth assays suggest an increased level of cell survival, after treatment with a DNA damage agent, when miR-20a is overexpressed in PC3 cells. Taken together, these experiments support the hypothesis that miR-20a has an anti-apoptotic function, possibly through its regulation of E2F1 expression.

DISCUSSION

The various programs of gene expression, which explain the myriads of cell phenotypes or their response to environmental
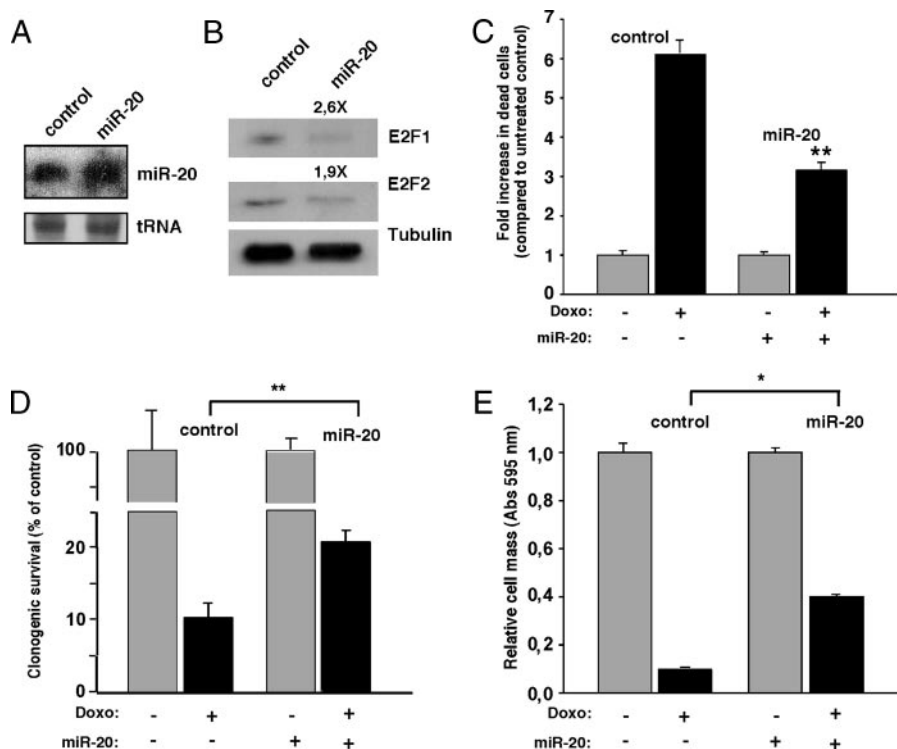
FIGURE 7. **miR-20a protects PC3 cells from doxorubicin-induced E2F-mediated cell death/apoptosis.** *A*, Northern blot on miR-20a miRNA from PC3 cells transfected with the control vector or the MLP-miR-20a plasmid. *B*, Western blot analysis of the levels of E2F1 and E2F2 in PC3 cells transfected with the control vector or the MLP-miR-20a plasmid. *Numbers* correspond to fold decrease compared with control. *C*, cell death of PC3 cells transfected with MLP-miR-20a or control vector after treatment with doxorubicin (100 ng/ml). Cell death was assessed using the Trypan blue exclusion assay. *D*, clonogenic survival of PC3 cells transfected with empty vector (*control*) or MPL-miR-20a vector after treatment with doxorubicin (12.5 ng/ml). Values correspond to the relative clone number compared with respective untreated cells. *E*, growth of PC3 cells transfected with empty vector (*control*) or MPL-miR-20a vector after treatment with doxorubicin (125 ng/ml). Values correspond to the relative absorbance compared with respective untreated cells. Paired *t* test: *, $p < 0.01$; **, $p < 0.05$.

FIGURE 8. **Model for an autoregulatory feedback loop between E2Fs and the *mir-17–92* cluster.** *A*, E2F1–3 are involved in a positive autoregulatory loop and stimulate the transcription of their own genes. Activation of the *mir-17–92* cluster by E2F1–3 would balance the positive autoregulatory loop of E2F1–3 by a negative feedback loop to control the level of expression of these transcription factors. *B*, double feed-forward loop between E2F1–3, Myc, and the miR-20a miRNA family. Since E2F1–3 activate the transcription of *MYC*, and vice versa (double-headed arrow), the transcriptional activation of the *mir-17–92* cluster by both transcription factors would maintain a level of miR-20a/miR-17 proportional to E2Fs activity.

gene regulation. These miRNAs can regulate gene expression both at transcriptional and post-transcriptional levels (20). It has been proposed that they can act as developmental switches or as fail safe regulators of transcriptional programs (33). Since miRNAs are themselves transcribed by RNA polymerase II, they can be regulated by transcriptional regulators. This fact predicts that interesting regulatory loops can be established between genes coding for classic transcription factors and genes coding for miRNAs.

We report here that the *mir-17–92* cluster is directly regulated by the E2F family of transcription factors. Since several miRNAs encoded in this cluster can repress E2F1–3 expression, an interesting autoregulatory feedback loop can be proposed between E2Fs and the *mir-17–92* cluster (Fig. 8*A*). It is well established that E2F1–3 are involved in a positive autoregulatory loop because they stimulate their own genes (17, 18). We propose that a role of the miR-20a miRNA family is to balance the positive autoregulatory loop of E2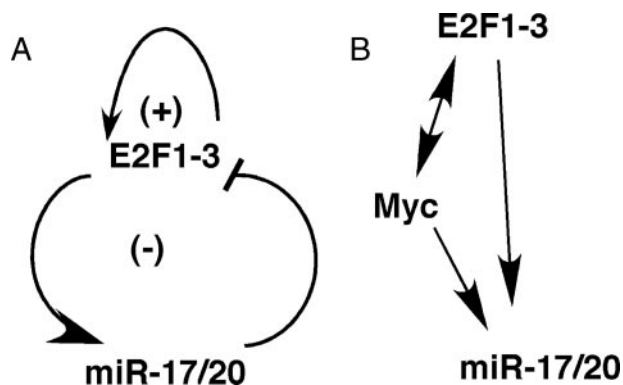F1–3 by a negative feedback loop to control the level of expression of these transcription factors. We further suggest that other transcription factors involved in positive autoregulatory loops may also be controlled by negative feedback loops involving miRNAs as transcriptional targets. An example may be the transcription factor MyoD, which is involved in myoblast differentiation and which is known to activate the transcription of its own gene (34). Recently, Lodish and colleagues (35) have shown that MyoD activates the transcription of the muscle specific miR-1 and miR-133 miRNAs. Interestingly, the MyoD mRNA has a potential miR-133 target site in its 3′-UTR (21), and it may be involved in a negative feedback loop that controls MyoD levels and activity. In the negative feedback loop between E2F1–3 and miR-20a, another layer of complexity must be added due to the activation of the *mir-17–92* cluster by c-Myc (19). Since E2F1–3 are known to activate the transcription of *MYC* (36), and c-Myc can activate the transcription of *E2F1–3* (37, 38), and both transcription factors activate the *mir-17–92* cluster, it suggest that this system may represent a novel variant of the feed-forward loop (39), which can be dubbed as a double feed-forward loop (Fig. 8*B*).

One interesting function of the negative feedback loop between E2Fs and the miR-20a family of miRNAs would be to create a fail-safe mechanism to avoid high E2F activity. High E2F activity is potentially dangerous for the cell, because it can lead to cell death or malignant transformation depending on

stimuli, have been classically viewed as the resultant of the combinatorial action of transcriptional regulators that can activate or repress specific genes. More recently, a family of small RNA molecules has emerged to challenge this accepted paradigm of

the cellular context. The physiological function of this circuit may be relevant to normal cell cycle regulation, where E2F1–3 levels can potentially reach high levels due to the well known fact that E2F1–3 positively regulate their own promoters. This also raises the possibility that the higher E2Fs levels observed with miR-20a inhibition may not come entirely from an increased E2Fs mRNA translation but may be caused in part by a secondary effect of the transcriptional up-regulation of the E2F1–3 genes by higher E2F activity. Our observations that miR-20a targets preferentially the 3′-UTR of the *E2F1* mRNA compared with the mRNAs of *E2F2* and *E2F3* suggest that E2F1 levels are more critical to cell survival, possibly due to the pro-apoptotic function of E2F1. This regulation may also be relevant to stem cell biology, where the Rb family is functionally inactivated by constitutive hyperphosphorylation and E2F activity is not subjected to Rb repression during the cell cycle (40). Interestingly, the *mir-17–92* cluster is highly expressed in mouse ES cells (41), which suggests the possibility that in the absence of functional Rb activity, E2F1–3 activity may be controlled instead by this negative feedback loop.

The E2F/miR20a autoregulatory feedback loop may be also operative during the transformation of normal cells into tumor cells. Since E2F1 is known to have both tumor suppressor and oncogenic properties depending on the cellular context, the miRNA fail-safe mechanism that prevents high E2Fs levels can therefore promote or suppress tumor formation. An oncogenic role for the miR-20a family of miRNAs is consistent with the anti-apoptotic role of this miRNA revealed in this study. Also, enforced expression of the *mir-17–92* cluster in a *Eμ-myc* mouse strain accelerated lymphomagenesis (23). While the anti-apoptotic functions of miR-20a may partly explain this oncogenic activity and the observed decrease in cell death in the *Eμ-myc* mouse overexpressing the *mir-17–92* cluster, a previous study has shown that myc-induced apoptosis in the *Eμ-myc* mouse is E2F1-independent (42). However, it is possible that other members of the *mir-17–92* cluster may target other pro-apoptotic genes. In addition, the genetic effects of miRNAs are different and subtler than the effects obtained in knock-out animals where gene expression is totally abolished. On the other hand, in other cell types, the miR-20a family may act as a tumor suppressor by preventing the proliferative activity of E2F1–3. In agreement, the *mir-17–92* cluster was found deleted in a high percentage of ovarian and breast cancers (43).

Our results contribute to the understanding of the complex regulatory pathways regulating E2F activity and are therefore relevant for studies on cell cycle regulation, cell death, and transformation. In addition, the fail-safe interactions between miRNAs and transcription factors described here may announce a much more common regulatory pattern.

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