# Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells

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## Abstract

***Background:*** **A major challenge in cancer chemotherapy has been developing safe and clinically efficacious chemotherapeutic agents. With its low toxicity profile, curcumin (diferuloylmethane), a naturally occurring flavinoid derived from the rhizome of *Curcuma longa*, has great promise. *In vitro* and *in vivo* preclinical studies have shown its inhibitory anticancer, antioxidant, anti-inflammatory, antiproliferative, and proapoptotic activities. The multiple mechanisms of the antitumor effect of curcumin putatively include down-regulating the expression of gene products such as nuclear factor-κB, growth suppression, inducing apoptosis, and modulating various signal transduction pathways and the expression of many oncogenes. The mechanisms underlying the antitumor activity of curcumin have not, however, been completely delineated. *Methods:* An oligonucleotide microarray chip was developed and used to profile microRNA (miRNA) expressions in pancreatic cells treated with curcumin. Transcripts with regulated expression patterns on the arrays were validated by real-time PCRs. Additionally, potential mRNA targets were analyzed bioinformatically and confirmed with flow cytometry experiments. *Results:* Curcumin alters miRNA expression in human pancreatic cells, up-regulating miRNA-22 and down-regulating miRNA-199a*, as confirmed by TaqMan real-time PCR. Upregulation of miRNA-22 expression by curcumin or by transfection with miRNA-22 mimetics in the PxBC-3 pancreatic cancer cell line suppressed expression of its target genes *SP1 transcription factor (SP1) and estrogen receptor 1 (ESR1)*, while inhibiting miRNA-22 with antisense enhanced SP1 and ESR1 expression. *Conclusions:* These observations suggest that modulation of miRNA expression may be an important mechanism underlying the biological effects of curcumin. [Mol Cancer Ther 2008;7(3):464–73]**

## Introduction

The chemotherapeutic agents long used in oncologic treatment produce deleterious side effects that augment the mortality and morbidity caused by cancer. Safer treatments are thus desperately needed, some of which can be found in natural compounds such as phytochemicals. Having established chemopreventive activities and preclinical antitumor effects, phytochemicals provide a novel therapeutic approach that merits further exploration (1, 2).

Clinically tested plant-derived anticancer agents with proven benefit include paclitaxel, etoposide and teniposide, *Vinca* alkaloids, vinblastine and vincristine, and camptothecin derivatives (1–4). These compounds share common antitumor activities and signaling pathways targeting the tumor cell cycle and cell death (5). Preclinical studies in diverse cancer cell lines, including breast, cervical, colon, brain, gastric, hepatic, leukemia, oral epithelial, ovarian, pancreatic, melanoma, and prostate, point to the anticancer activity of curcumin (diferuloylmethane; refs. 6–11), a naturally occurring flavinoid and proapoptotic compound derived from the rhizome of *Curcuma longa*. Liposomal-encapsulated curcumin, which can be given i.v. (and therefore circumvents the problem of poor absorption of oral curcumin), shows antitumor effects in animal models of pancreatic and colorectal cancer (9, 10, 12). Curcumin also potentiates the effects of chemotherapy in animal models (12) and has shown safety and tolerability in doses as high as 8,000 mg/d in patients. As yet, no maximum tolerated dose has been established (13–15).[4] There is a wealth of experimental evidence suggesting that multiple mechanisms of action are likely responsible for the various pharmacologic effects of curcumin on cancer-related signaling molecules (9, 10, 12–20). These include modulation of Jak/STAT, nuclear factor-κB, extracellular signal-regulated kinase, jun, cyclooxygenase, and other key molecules involved in tumorigenesis.

[4] N. Dhillon, B.B. Aggarwal, R.A. Newman, et al. Phase II trial of curcumin, an NF-κB inhibitor, in patients with advanced pancreatic cancer, submitted for publication.

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MicroRNAs (miRNA) are highly conserved, small noncoding RNAs composed of 20 to 22 nucleotides encoded in the genomes of plants and animals. They are typically excised from 60 to 110 nucleotide fold-back RNA precursor structures (for reviews, see refs. 21–23). Although identified relatively recently, the small but important miRNAs have been recognized as comprising one of the major regulatory gene families in eukaryotic cells, and it has been speculated that their role in regulating gene expression in higher eukaryotes could rival that of transcription factors. miRNAs are involved in crucial biological processes, including development, differentiation, apoptosis, and proliferation (22, 24, 25) through imperfect pairing with target mRNAs of protein-coding genes and the transcriptional or posttranscriptional regulation of their expression (26–28). Evidence is emerging rapidly that specific miRNAs might play a role in human cancer pathogenesis. Various cancers have been associated with both their down-regulation (29–33) and up-regulation (34–36). However, very few compounds, not to mention cancer drugs, which affect cell growth and/or development, have been shown to affect miRNA expression (37).

We developed a sensitive microarray chip for miRNA expression profiling, which we used in the current study to test our hypothesis that curcumin alters miRNA expression profiles. Our results indicated that curcumin alters specific miRNA expression in human pancreatic cells. Furthermore, gene expression that is targeted by these specific miRNAs down-regulated by curcumin was also modulated by curcumin. It therefore appears that miRNA-related changes are an important effect of curcumin.

## Materials and Methods

### Cell Lines

The BxPC-3 human pancreatic carcinoma cell line was purchased from American Type Culture Collection and was cultured according to the manufacturer's instructions. Specifically, the cells were grown in RPMI 1640 supplemented with 10% FCS. RPMI 1640 was purchased from Invitrogen. Cells were seeded at a concentration of $5.0 \times 10^5$ per 100-cm plate and incubated overnight. These cells were subsequently treated with various concentrations of curcumin dissolved in DMSO (final DMSO concentration in growth media, 0.1%). After a 72-h incubation period, the cells were trypsinized and counted. For each treatment, identical numbers of cells ($3.0 \times 10^3$-$5.0 \times 10^3$ per well) were seeded in triplicate on 96-well plates in curcumin-free medium and incubated for 72 h.

### RNA Isolation

We extracted miRNA-enriched total RNAs using Ambion's microRNAVana miRNA Isolation Kit following the manufacturer's protocol with modifications. Briefly, $10^2$ to $10^7$ cells were collected and washed with cold PBS buffer. The cells were disrupted in lysis/binding buffer and vortexed briefly. About 1:10 volume of miRNA homogenate additive was added to the cell lysate and the mixture was incubated on ice for 10 min. The sample lysate was extracted with an equal volume of acid/phenol/chloroform and mixed with a 1.25 volume of 100% ethanol. The lysate/ethanol mixture was passed through the filter cartridge. RNA was eluted with either elution solution or nuclease-free water after the filter cartridge was washed with miRNA washing solution. To increase RNA quality, the eluted RNA was loaded onto a new filter cartridge and washed a second time with washing buffers. The RNA eluted from the second filter cartridge was used for microarray experiments after a quality assurance test was done using an Agilent Bioanalyzer.

### miRNA Microarray

**miRNA Sequences.** We downloaded all *Homo sapiens* miRNA precursors and mature miRNAs from the miRNA registry at Rfam (release 5.0).[5] For each miRNA, we checked sequence uniqueness to avoid redundancy by blasting each sequence against our miRNA sequence database. Our miRNA database was also updated manually by collecting new miRNA information from recently published papers.

**Microarray Design.** Our miRNA microarrays are designed based on a proprietary uParaflo microfluidic technology developed by Atactic Technologies. The technology allows parallel synthesis of a large number of different oligonucleotide molecules at high yield without the need for the expensive, inconvenient microfabricated photomasks used previously. The chips contain the following probes: (*a*) Detection probes: Detection probes include all human miRNANA (antisense) sequences from the current version online.[6] Detection probes are composed of modified oligonucleotides, including all miRNANA (antisense) sequences. Ethylene glycol spacers were used between oligonucleotide sequences and the substrate surface. (*b*) Positive control probes: Two positive control probes were also used. The first positive control probe has a complementary sequence with a positive control RNA to be added into the total RNA sample. This first positive control is used to determine the quality of the total RNA in the analyzed sample. It is used in combination with a second positive control to assess sample preparation and labeling processes. The second positive control probe has a complementary sequence with a labeled (oligonucleotide) RNA sample to be added to the hybridization mix. This second positive control is used to reveal any obvious problems that are related to chip hybridization. Both positive controls are made of synthetic RNA. Their sequences were selected from Puc2 oligonucleotides, which were tested and found to have low cross-hybridization with most of the probes designed for mRNA detection. (*c*) Negative controls: Candidates for negative controls include miRNA sequences from different species, antisense human miRNA oligonucleotides (probes are sense miRNA sequences), and artificially designed sequences for reference purposes. We randomly generated ten 22-nucleotide-long oligonucleotide

[5] http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml
[6] http://www.sanger.ac.uk/Software/Rfam/microRNAna/

sequences with no significant similarity to any of the genome assemblies and included these sequences on the microarray chip. In case we had fewer than 10 human miRNANAs with no expression in our testing sample, we made spiking sequences for Latin square tests by choosing select sequences from tested negative control sequences. (*d*) Reference probes: We selected two housekeeping genes, *$\beta_2$-microglobulin* and *hypoxanthine phosphoribosyltransferase 1*, to generate reference probes. tTNA probes were used to detect reference sequences for normalizing mRNA levels. Process control probes—hybridization stringency: At least two sets of hybridization stringency control probes were added. These test probes contain substitution, insertion, and deletion mismatches from the two positive control sequences. Perfect match to mismatch ratios are used to assess hybridization stringency.

### Each Probe Sequence Was Repeated Six Times on the Same Array Chip

**Sample Labeling.** Dendrimer Cy3 and Cy5 dyes were used to label RNA sequences to enhance visualization. First, poly(A) tails were added to the 3′ ends of RNA sequences using poly(A) polymerase followed by the addition of a nucleotide tag to the poly(A) tail via a ligation reaction. For dual-sample experiments, the two sets of RNA sequences are added with tags from both sequences. The tagged RNA sequences are then hybridized to the array. Labeling is carried out during the second hybridization reaction using commercially available tag-specific dendrimer Cy3 and Cy5 dyes.

**Array Hybridization.** Labeled targets from 5 μg total RNA were used for hybridization on each Phase I Program/M. D. Anderson miRNA microarray chip containing 300 probes with six replicates each. All probes on these microarrays are 40-mer oligonucleotides spotted by contacting technologies and covalently attached to a polymeric matrix. The microarrays were hybridized in 6× SSPE [0.9 mol/L NaCl/60 mmol/L sodium phosphate/8 mmol/L EDTA (pH 7.4)], 30% formamide at 25°C for 18 h, washed in 0.75× Tris-HCl/NaCl/Tween 20 at 37°C for 40 min, and processed using direct detection of the biotin-containing transcripts by streptavidin-Alexa 647 conjugate. Processed slides were scanned using a Perkin-Elmer ScanArray XL5K Scanner with the laser set to 635 nm, at power 80 and PMT 70 settings, with a scan resolution of 10 μm.

### Quantitative Real-time PCR for miRNA Analysis

Applied Biosystems TaqMan miRNA assays are designed to detect and quantify mature miRNAs using looped-primer real-time PCR. The human early access panel used in this study contained 160 individual assays covering many of the identified human miRNAs. The assay involved two steps: (*a*) stem-looped primers for reverse transcription of the miRNA are followed by real-time PCR. Briefly, single-stranded cDNA was generated from the total RNA sample by reverse transcription using the Applied Biosystems High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer's protocol. Reverse transcription reactions contained 10 ng total RNA, 50 nmol/L stem-looped reverse transcription primer, 1× reverse transcription buffer, 0.25 mmol/L each of dNTPs, 3.33 U/Ol MultiScribe reverse transcriptase, and 0.25 U/Ol RNase inhibitor. PCR amplification was carried out using sequence-specific primers on the Applied Biosystems 7500 Real-time PCR System. The reactions were incubated in a 96-well optical plate at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 10 min. Relative miRNA expression data were analyzed using the ΔÄCT method with hsa-5s RNA as an endogenous control.

### miRNA Transfection

**Transfection Reagent and RNA Oligonucleotides.** Transfection reagent (DharmaFECT 1) was purchased from Dharmacon. miRNA oligonucleotide, miRIDIAN miRNA inhibitor, has-miR-22, and miRNA mimics were also purchased from Dharmacon. The miRNA inhibitors were synthesized and single-stranded miRNA oligonucleotides, which specifically target and irreversibly bind targeted miRNA molecules, were examined. miRNA mimics were synthesized and dsRNA was designed to mimic the function of endogenous mature miRNA while minimizing the IFN response. The active strand preferentially enters the miRNA pathway to up-regulate the expression level of the targeted miRNA. In addition, siGLO Green Transfection Indicator was purchased from Dharmacon for simplified transfection assessment without doing a functional assay.

**Cell Plating.** Cells growing on flasks were trypsinized and counted. Cells were diluted with antibiotic-free medium to an appropriate concentration for the transfection experiments. Cells (100 μL) were plated in each well of a 96-well culture plate and incubated at 37°C with 5% $CO_2$ overnight.

**Transfection.** Solutions (2 μmol/L) of miRIDIAN miRNA inhibitors and miRNA were prepared by adding 1× small interfering RNA buffer. Dilute 2 μmol/L small interfering RNA was placed in tube 1 and the transfection reagent was placed in tube 2 with serum-free medium. The diluted miRNA and transfection reagent were mixed gently and incubated for 5 min at room temperature. The contents in tubes 1 and 2 were combined and mixed by pipetting the solution carefully up and down a few times and then incubated for 20 min at room temperature. Complete medium (80 μL) was added to the mix, which was then added to each well of a 96-well plate after removing the growth medium. The transfected cells were incubated at 37°C with 5% $CO_2$ for 48 h.

**Prediction of miRNA Targets.** To identify the targets of human miRNAs, we used PicTar[7] and TargetScan[8] software by following the manufacturer's instructions.

**Flow Cytometry Analysis.** Antibodies for Sp1 transcription factor (SP1; GTX13370) and estrogen receptor (GTX21104) were purchased from GeneTex.

Fluorescence-activated cell sorting was done to quantify target protein expression. Briefly, after transfection or

[7] http://pictar.bio.nyu.edu
[8] http://genes.mit.edu/targetscan

curcumin treatment, cells were fixed in 2% paraformaldehyde/PBS for 10 min at 37°C and permeabilized in 90% methanol for 30 min on ice. The cells were then incubated with the first antibody and with the second antibody in PBS plus 2% fetal bovine serum for 60 min, respectively.

Cells were acquired using a flow cytometer and analyzed with the accompanying software (FACSCalibur and CellQuest, both from Becton Dickinson Immunocytometry Systems). The analysis was done by gating on SP1 or estrogen receptor 1 (ESR1) progenitor cell populations. Approximately 1,000 cells were acquired in each gated region. The percentages of cells expressing SP1 or ESR1 were calculated. The percentage of positive cells was calculated by subtracting the value of the appropriate isotype controls.

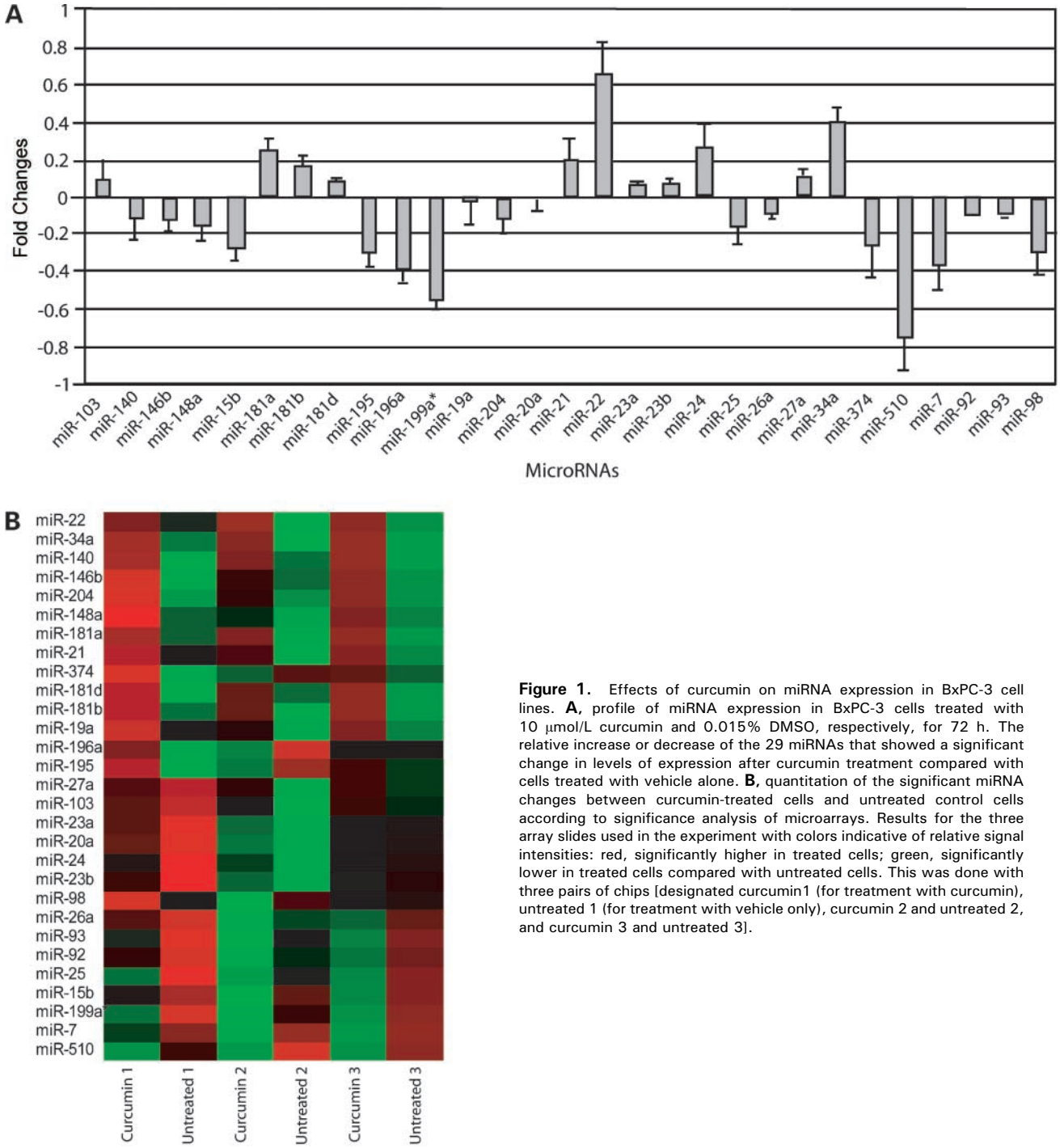


**Figure 1.** Effects of curcumin on miRNA expression in BxPC-3 cell lines. **A,** profile of miRNA expression in BxPC-3 cells treated with 10 μmol/L curcumin and 0.015% DMSO, respectively, for 72 h. The relative increase or decrease of the 29 miRNAs that showed a significant change in levels of expression after curcumin treatment compared with cells treated with vehicle alone. **B,** quantitation of the significant miRNA changes between curcumin-treated cells and untreated control cells according to significance analysis of microarrays. Results for the three array slides used in the experiment with colors indicative of relative signal intensities: red, significantly higher in treated cells; green, significantly lower in treated cells compared with untreated cells. This was done with three pairs of chips [designated curcumin1 (for treatment with curcumin), untreated 1 (for treatment with vehicle only), curcumin 2 and untreated 2, and curcumin 3 and untreated 3].

### Bioinformatics and Statistical Analysis

The analyses included data preprocessing and determination of differentially expressed genes. The first preprocessing procedure was background correction calculated by subtracting median background from median intensity for each spot. Secondly, a global normalization was done by setting the 75th percentile to 1,000 for each individual channel. In addition, the normalized values were truncated at a minimum value of 25. In the end, all the measurements were transformed by using a base 2 log.

After the above preprocessing, we plotted gene expression measurements in the green channel against the ones in the red channel within each array. These plots examined whether the expression measurements from the same genes were consistent under the two channels. If they were, the plots should exhibit straight lines. However, we observed some curvatures for our data, indicating that some systemic trend between the two channels still existed and further normalization was needed. Thus, we subsequently did normalization using local linear regression models on the gene expression measurements from the two channels, after which the trend was removed and all the plots exhibited straight lines.

After the normalization, to determine the effects of curcumin and liposomal curcumin on gene expression, we fitted an ANOVA model to examine whether genes were differentially expressed between treatment and control conditions. We obtained $P$ values for all the genes after doing these analyses and obtained lists genes with the largest differential expression between each treatment and control conditions.

**Databases Blasted and Genome Analysis.** The majority of the miRNAs used in these experiments were found in the miRNA databases.[9,10] Others were collected from published papers. PERL modules[11] and BIOPERL modules[12] were used to facilitate database blast and genome analysis. All statistical analyses were done in S-Plus (Insightful Corp.). After omitting blanks and negative controls, spot intensities on each miRNA microarray were background corrected by subtracting the 15th percentile of observed intensity. Log-transformed, background-corrected intensities between Cy3 and Cy5 channels of each array were Loess normalized.

## Results

### Curcumin Treatment Alters miRNA Expression Profiles

To study the responses of miRNAs to curcumin, microarray analysis of miRNA expression was conducted with miRNA-enriched total RNAs extracted from BxPC-3 pancreatic cells treated with different concentrations of curcumin or liposomal curcumin. RNA samples were processed, labeled, and hybridized to Atactic miRNA chips as described in Materials and Methods. Cluster analysis was conducted on the expression profiles of the curcumin-treated and liposomal curcumin-treated BxPC-3 cells at concentrations of 0 and 10 μmol/L. Three miRNA microarray chips were used in the current experiment. Figure 1*A* and *B* displays relative indocarbocyanine (C3) dye intensity

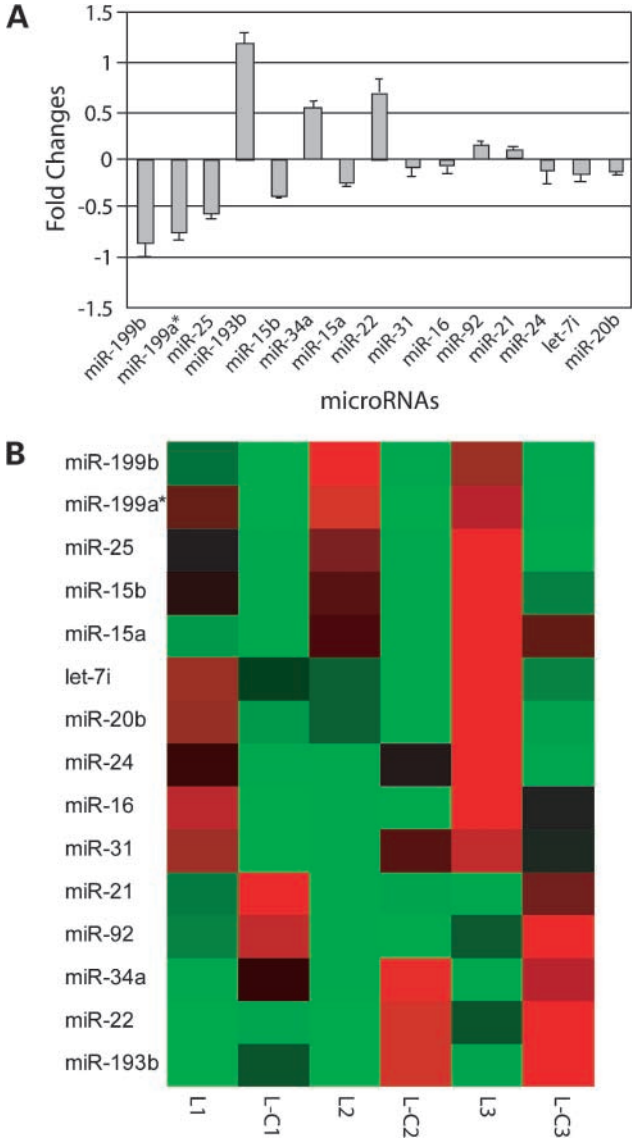


**Figure 2.** Effects of liposomal curcumin treatments on miRNA expression in BxPC-3 cell line. **A**, profile of miRNA expression in BxPC-3 cells treated with 10 μmol/L liposomal curcumin and liposome, respectively, for 72 h. The relative increase or decrease of the 15 miRNAs that showed a significant change in levels of expression after liposomal curcumin treatment compared with cells treated with empty liposomes alone. **B**, quantitation of significant miRNA changes between liposomal curcumin treatment and its liposomal control cells according to significance analysis of microarrays analysis. Results for the three array slides used in the experiment with colors indicative of relative signal intensities: red, significantly higher in treated cells; green, significantly lower in treated cells compared with untreated cells. This was done with three pairs of chips [designated L1 (for treatment with empty liposomes), LC-1 (for treatment with liposomal curcumin), L2 and LC-2, and L3 and LC-3].

[9] http://www.sanger.ac.uk/Software/Rfam
[10] http://www.ncbi.nlm.nih.gov
[11] http://www.perl.com
[12] http://bio.perl.org

levels. Only miRNAs with significant expression over background and those that showed a differential expression among curcumin or liposomal curcumin treatments and their controls are shown. Figure 1 shows the effects of 10 μmol/L curcumin on miRNA expression in BxPC3 cells. After 72-h incubation, 11 miRNAs were significantly up-regulated, whereas 18 were significantly down-regulated by curcumin compared with its DMSO control ($P \leq 0.05$; Fig. 1*A* and *B*). Among these miRNAs, miRNA-22 was up-regulated by 65.5%, whereas miRNA-199a* was down-regulated by 54.2%. Similarly, at the same concentration (10 μmol/L), liposomal curcumin significantly up-regulated the expression of 5 miRNAs and down-regulated 10 miRNAs ($P \leq 0.05$; Fig. 2*A* and *B*). Liposomal curcumin (10 μmol/L) enhanced miRNA-22 expression (68%) but decreased miRNA-199a* expression (71%). A greater number of miRNAs were down-regulated than up-regulated for both treatments in both cases. Interestingly, miRNA-22 was up-regulated and miRNA-199a* was down-regulated consistently and substantially by both curcumin and liposomal curcumin ($P \leq 0.05$; Figs. 1 and 2).

### Confirmatory Studies with Differentially Expressed miRNAs by TaqMan Real-time PCR

For comparison purpose, the effects of curcumin and liposomal curcumin (10 μmol/L) on miRNA-22 and miRNA-199a* are presented in Fig. 3*A*. Quantitative real-time PCR was done for the two miRNAs, miRNA-22 and miRNA-199a*, which were up-regulated and down-regulated, respectively, by curcumin and liposomal curcumin in the microarray experiments (Fig. 3*A*). miRNA-enriched total RNAs from the same preparation used for microarray analysis were reverse-transcribed and amplified in the ABI Prism 7500 Sequence Detection System for TaqMan analysis. As shown in Fig. 3, miRNA-22 was up-regulated by 10 μmol/L curcumin and 10 μmol/L liposomal curcumin by 60.3% and 68.6%, respectively (Fig. 3*B*), whereas miRNA-199a* was down-regulated by 25.1% and 36.40% (Fig. 3*C*), respectively. The results obtained by quantitative real-time PCRs were comparable with and confirmed the microarray data (Fig. 3*A*), although miRNA-199a* was down-regulated to a lesser degree in the TaqMan analyses (Fig. 3*C*).

### Predictions of the Target Genes of miRNA-22 and miRNA-199a*

The functional characterization of miRNAs relies heavily on the identification of their target mRNAs. The major challenge for current miRNA studies is to identify the biologically relevant targets that they regulate. Toward this end, various bioinformatic and combined bioinformatic and experimental approaches have been done to identify putative target miRNAs. Several computer programs, including PicTar[13] and TargetScan,[14] have been developed for genome-wide searches of miRNA targets, which, following the manufacturer's instructions, we used to identify the targets of miRNAs.

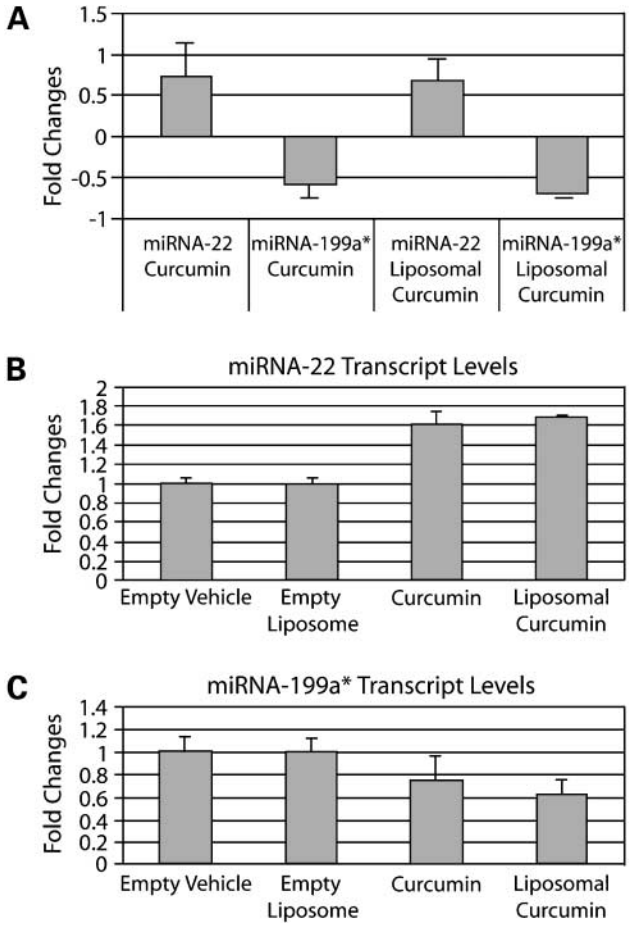


**Figure 3.** Comparison of microarray analysis with real-time PCR on the expressions of miRNA-22 and miRNA-199a*. BxPC-3 cells were incubated for 72 h with 10 μmol/L liposomal curcumin or controls. **A,** microarray analysis. The fold changes of curcumin-regulated or liposomal curcumin-regulated miRNA-22 and miRNA-199a* compared with untreated controls. **B,** real-time PCR confirmation of miRNA-22 expression. Columns, mean fold; bars, SE ($n = 4$). *, $P < 0.05$, compared with normal control values in the absence of curcumin. **C,** real-time PCR confirmation of miRNA-199a* expression. Columns, mean fold; bars, SE ($n = 4$). *, $P < 0.05$, compared with normal control values in the absence of curcumin.

Two discrete lists of specific target genes were generated from PicTar and TargetScan. A third list was composed of the genes that were common to both of those lists. The most promising 50 candidate genes for miRNA-22 are listed in Table 1. In consideration of the false-positive rates from both applications, ESR1 and SP1, potential target genes identified by PicTar and TargetScan, were chosen for supplementary studies because their functions are already known and could be discriminated from the results of further experiments with miRNA-22.

### SP1 and ESR1 Regulation by miRNA-22

We wanted to determine whether SP1 and ESR1 could be manipulated by up-regulating and/or down-regulating miRNA-22. miRNA oligonucleotide, miRIDIAN miRNA-22

[13] http://pictar.bio.nyu.edu
[14] http://genes.mit.edu/targetscan

inhibitors and mimics were transfected into BxPC-3 cells. The miRNA inhibitors, single-stranded chemically enhanced oligonucleotides, specifically target and irreversibly bind targeted miRNA molecules. miRNA mimics are synthesized dsRNA that are designed to mimic the mature miRNA pathway while minimizing an IFN response. The active strand preferentially enters the miRNA pathway to up-regulate the expression of the targeted miRNA. Cells were stained with the ESR1 and SP1 antibodies 72 h after 10 μmol/L curcumin treatment and 48 h after transfection, respectively. The expression levels of ESR1 and SP1 proteins were determined from the use of flow cytometry and the results are summarized in Figs. 4 and 5, respectively.

Treating BxPC-3 cells with 10 μmol/L curcumin down-regulates both ESR1 (48.6%; Fig. 4*A*) and SP1 (42.4%; $P \leq 0.05$; Fig. 5*A*). Transfection of miRNA-22 oligonucleotides significantly altered both ESR1 and SP1 ($P \leq 0.05$; Figs. 4*B* and 5*B*). When miRNA-22 was inhibited by transfecting the antisense RNA oligonucleotide of miRNA-22 into BxPC-3 cells, ESR1 and SP1 proteins were significantly up-regulated by 196% and 132%, respectively ($P \leq 0.05$). However, transfecting the RNA oligonucleotide of miRNA-22 down-regulated ESR1 and SP1 by 65% and 61%, respectively ($P \leq 0.05$; Figs. 4*B* and 5*B*). Because the expression of the target genes, *ESR1* and *SP1*, could be manipulated by up-regulating and down-regulating miRNA-22, it seems logical to conclude that miRNA-22 has an important role in regulating gene expression that may rival the significance of transcription factors.

## Discussion

Complex regulatory networks are responsible for the expression of genes in normal cells. When these networks are perturbed, cancer is one result. It is clear from recent studies that the abundant existence of small non-protein-coding regulatory RNAs, known as miRNAs, is important in cancer (30, 31). miRNAs have been shown in preclinical

**Table 1. Target genes of miRNA-22**

| Target gene | Accession no. | Target gene name | Target gene | Accession no. | Target gene name |
|---|---|---|---|---|---|
| *ESR1* | NM_000125 | *Estrogen receptor 1* | *KIAA0427* | NM_014772 | *KIAA0427* |
| *BCL9L* | NM_182557 | *B-cell CLL/lymphoma 9-like* | *ODF1* | NM_024410 | *Outer dense fiber of sperm tails 1* |
| *LAMC1* | NM_002293 | *Laminin, γ1 precursor* | *TTYH3* | NM_025250 | *Tweety 3* |
| *FAM49B* | NM_016623 | *Family with sequence similarity 49, member B* | *ARID3B* | NM_006465 | *AT-rich interactive domain 3B (BRIGHT-like)* |
| *H3F3B* | NM_005324 | *H3 histone, family 3B* | *MXD4* | NM_006454 | *MAD4* |
| *NUDT4* | NM_199040 | *Nudix (nucleoside diphosphate-linked moiety)* | *YARS* | NM_003680 | *Tyrosyl-tRNA synthetase* |
| *CAV3* | NM_001234 | *Caveolin 3* | *RFXANK* | NM_003721 | *Regulatory factor X-associated* |
| *RAPGEFL1* | NM_016339 | *Rap guanine nucleotide exchange factor* | *TNKS2* | NM_025235 | *Tankyrase, TRF1-interacting ankyrin related* |
| *FLJ14936* | NM_032864 | *Hypothetical protein FLJ14936* | *FOSL1* | NM_005438 | *FOS-like antigen 1* |
| *FLJ14936* | NM_032284 | *Hypothetical protein FLJ14936* | *TYRO3* | NM_006293 | *TYRO3 protein tyrosine kinase* |
| *OGN* | NM_033014 | *Osteoglycin preproprotein* | *DPF2* | NM_006268 | *D4, zinc and double PHD fingers family 2* |
| *RAB5B* | NM_002868 | *RAB5B, member RAS oncogene family* | *RYR1* | NM_000540 | *Ryanodine receptor 1 (skeletal)* |
| *NAT5* | NM_181527 | *N-acetyltransferase 5 isoform a* | *MAP3K3* | NM_203351 | *Mitogen-activated protein kinase kinase kinase 3* |
| *PPARA* | NM_001001929 | *Peroxisome proliferative-activated receptor* | *SP1* | NM_138473 | *Sp1 transcription factor* |
| *IL13RA1* | NM_001560 | *Interleukin-13 receptor, α1 precursor* | *MECP2* | NM_004992 | *Methyl CpG-binding protein 2* |
| *CYLN2* | NM_032421 | *Cytoplasmic linker 2 isoform 2* | *EPC1* | NM_025209 | *Enhancer of polycomb 1* |
| *C21orf4* | NM_006134 | *HCV p7-transregulated protein 3* | *PLAGL2* | NM_002657 | *Pleiomorphic adenoma gene-like 2* |
| *EMILIN3* | NM_052846 | *Elastin microfibril interfacer 3* | *WASF1* | NM_003931 | *WAS protein family, member 1* |
| *MUC4* | NM_138298 | *Mucin 4 isoform a* | *NR3C1* | NM_000176 | *Nuclear receptor subfamily 3, group C, member 1* |
| *FLJ21128* | NM_025083 | *Hypothetical protein FLJ21128* | *COPS7B* | NM_022730 | *COP9 constitutive photomorphogenic homologue* |
| *IL17RD* | NM_017563 | *Interleukin-17 receptor D* | *FGFR2* | NM_023028 | *Fibroblast growth factor receptor 2 isoform 5* |
| *PRR6* | NM_181716 | *Proline rich 6* | *FGFR2* | NM_023030 | *Fibroblast growth factor receptor 2 isoform 5* |
| *TP53INP1* | NM_033285 | *Tumor protein p53-inducible nuclear protein 1* | *FLJ22313* | NM_022373 | *Hypothetical protein FLJ22313* |
| *SATB2* | NM_015265 | *SATB family member 2* | *LAT1-3TM* | NM_031211 | *LAT1-3TM protein* |
| *LGALS1* | NM_002305 | *β-galactosidase binding lectin precursor* | *TLK2* | NM_006852 | *Tousled-like kinase 2* |

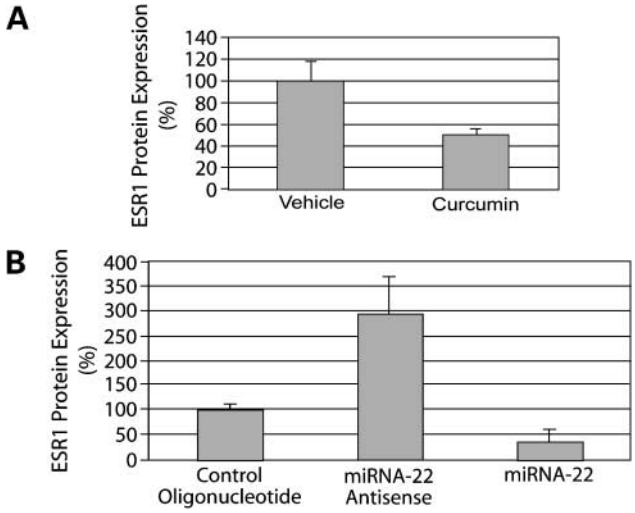


**Figure 4.** Analysis of ESR1 and SP1 expression in BxPC-3 cells by flow cytometry. Columns, mean fold; bars, SE ($n = 4$). **A,** effect of curcumin on ESR1 protein expression. BxPC-3 cells were treated with/without 10 μmol/L curcumin for 72 h. **B,** effect of miRNA-22 on ESR1 protein expression. BxPC-3 cell pools transiently transfected with either fluorescence-labeled oligonucleotide as a positive control, antisense miRNA-22 oligonucleotide, or sense miRNA-22 oligonucleotide. The cell pools were analyzed for enhanced green fluorescent protein by flow cytometry. The fluorescence recorded for untransfected control cells corresponds to autofluorescence. Arithmetic mean of fluorescence intensity was 3.4 (untransfected cells).

experiments to regulate diverse biological processes, including development, cell proliferation, differentiation, and apoptosis. These effects occur through the suppressed expression of key target genes. Posttranscriptional silencing of target genes by miRNAs occurs either by cleavage of homologous target mRNAs or by inhibition of target protein synthesis. Computational predictions indicate that one miRNA may target hundreds of genes and that more than 50% of human protein-coding genes could be regulated by miRNAs (38). Armed with this knowledge, miRNAs are receiving increased attention in cancer genomic research. It is becoming apparent that miRNAs can act as both oncogenes and/or tumor suppressor genes within the molecular architecture of gene regulatory networks, thereby contributing to the development of cancer. Importantly, for the future of personalized medicine and cancer treatment, miRNAs may provide useful diagnostic and prognostic markers for cancer diagnosis and treatment as well as serving as potential therapeutic targets or tools.

We have shown previously that curcumin has potent growth-inhibitory and proapoptotic effects on pancreatic cancer *in vitro* and *in vivo* (8–10). These effects may be mediated through any of the many ways that curcumin interferes with cell signaling (9, 10, 12–20). The goal of this study was to determine whether curcumin alters the expression profiles of miRNAs in human pancreatic cancer cells. Our microarray experiments showed that curcumin up-regulated the expression of some miRNAs (such as miRNA-22) but down-regulated others (such as miRNA-199a*). Real-time PCR experiments with miRNA-22 and miRNA-199a* further confirmed the expression patterns we found in our microarray experiments. We used PicTar and TargetScan to predict the target genes of miRNA-22. ESR1 and SP1 were chosen for further studies. Manipulating the expression of miRNA-22 by transfecting its miRNA oligonucleotides changed the expression of its downstream genes, *ESR1* and *SP1* (Fig. 5).

The transcriptional activity of ESR1 protein is implicated as having an especially important role in the estrogen nuclear pathway; consequently, ESR1 is one of the most important therapeutic targets in breast cancer. The relationship between ESR1 expression and cellular responsiveness to estrogens and antiestrogens has been extensively studied in cell lines, animal models, and humans (39–42). More than two-thirds of breast cancers show estrogen receptor expression at the time of diagnosis (43), and immunohistochemical detection of estrogen receptor expression is routinely used for making decisions involving hormonal therapy (antiestrogen receptor) for breast cancer (44). A recent analysis of more than 2,000 clinical samples showed a significant relationship between *ESR1* gene amplification and breast cancer (45). Current antiestrogen receptor treatment strategies include blocking its expression by selective modulators (such as tamoxifen and raloxifene), estrogen receptor destabilization and degradation via selective down-regulators (such as fulvestrant), and perturbing estrogen synthesis (aromatase inhibitors, such as anastozole, letrozole, or exemestane). These strategies substantially decrease tumor growth in ~30% to 50% of estrogen receptor–positive patients (46).

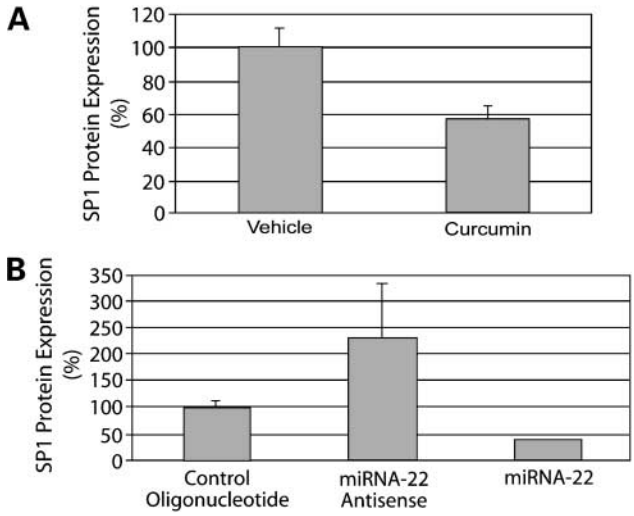


**Figure 5.** Analysis of SP1 expression in BxPC-3 cells by flow cytometry. **A**, effect of curcumin on SP1 protein expression. BxPC-3 cells were treated with/without 10 μmol/L curcumin for 72 h. **B,** effect of miRNA-22 on SP1 protein expression. BxPC-3 cell pools transiently transfected with either fluorescence-labeled oligonucleotide as a positive control, antisense miRNA-22 oligonucleotide, or sense miRNA-22 oligonucleotide. The cell pool was analyzed for enhanced green fluorescent protein fluorescence by flow cytometry. The fluorescence recorded for untransfected control cells corresponds to autofluorescence. Arithmetic mean of fluorescence intensity was 3.4 (untransfected cells).

The current study showed that the expression of ESR1 was decreased by ~50% in BxPC-3 cells treated with 10 μmol/L curcumin (Fig. 5*A*). Curcumin up-regulated miRNA-22 (Figs. 1-3) and computer analysis and transfection experiments suggested that ESR1 is one of its putative targets (Figs. 4 and 5). We hypothesize that one of the important effects of curcumin on cancer cells is as follows: Curcumin →Cancer →Cell miRNA-22 ↑→ESR1↓–→Tumorigenesis↓.

Of substantial interest in this regard is the fact that estrogen receptors have recently been found to be of physiologic importance in other tumors, including, but not limited to, ovarian, lung, and pancreatic cancer, and the potential for the use of estrogen modulating drugs in these malignancies is being investigated (47–50).

The SP1 family of transcription factors bind GC/GT-rich promoter elements through three C((2))H((2))-type zinc fingers in their COOH-terminal domains (51). SP1 protein regulates multiple genes in normal tissues and tumors (51). There is growing evidence that some Sp proteins have critical roles in growth and metastases in diverse tumor types (such as pancreatic cancer) by regulating cell cycle gene expression and vascular endothelial growth factor receptor (51, 52). Transcription factors such as SP1 are now recognized targets for the development of new anticancer drugs (51, 52), including drugs for pancreatic cancer (53). Curcumin down-regulated SP1 protein in the current study (Fig. 5*A*), which we believe occurs through miRNA-22 up-regulation (Fig. 5*B*).

Interestingly, a recent study showed that ERRα1, an estrogen-related receptor, stimulated the formation of a SP1-DNA complex as well as the induction of SP1-dependent luciferase activity (54). In addition, SP1 expression was up-regulated at the transcriptional level by ERRα1 transfection. The study found an ERRα1 binding site in the SP1 promoter region and showed that binding to this site was regulated by the A/B domain of ERRα1 (55). Similarly, ESR1 and SP1 coactivation is well documented (56–59). For example, vitamin D receptor interacting protein (DRIP150) coactivates ESR1 and SP1 in breast cancer cell lines (58). It is reasonable to predict that inactivating one may suppress the expression of the other. Here, we found that ESR1 and SP1 were simultaneously suppressed by curcumin (Figs. 4 and 5), although it is not clear whether one of the two genes modulates the other or if they are suppressed by a common upstream mechanism (such as miRNA).

Very few compounds have been known to alter miRNA expression profiles. In one study, the potent histone deacetylase inhibitor, LAQ824, rapidly altered cellular miRNA levels in SKBr3 breast cancer cells (37). Our study is the first to show that curcumin alters miRNA expression in cancer cells.

In conclusion, we used microarray technology to analyze the expression of all known human miRNAs following treatment with curcumin and liposomal curcumin. Two curcumin-responsive miRNAs, miRNA-22 and miRNA-199a*, showed major and consistent effects, which were validated by microarray analysis and real-time PCR. Furthermore, manipulating the expression of miRNA-22 altered the protein expression of its target genes, *ESR1* and *SP1*. This suggests an important and novel new mechanism by which curcumin mediates its potent effects on cell growth and apoptosis.

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