Supporting Information:

Figure S6.

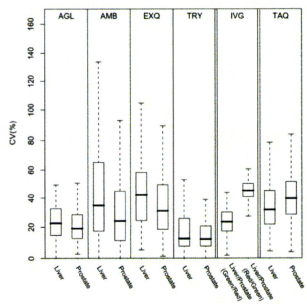
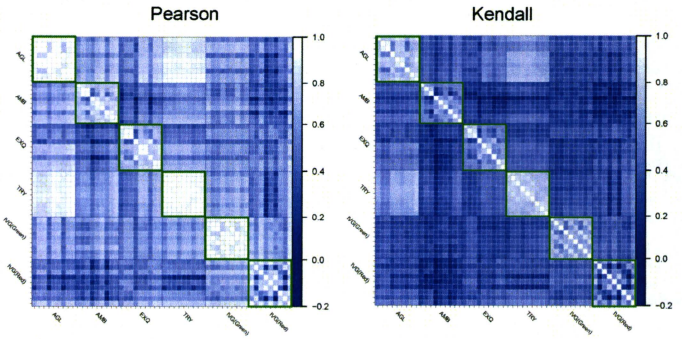


Figure S7.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MicroRNA-210 Regulates Cancer Cell Proliferation through Targeting Fibroblast Growth Factor Receptor-like 1 (*FGFRL1*)*[S]

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The importance of microRNAs (miRNAs) in human malignancies has been well recognized. Here, we report that the expression of microRNA-210 (miR-210) is down-regulated in human esophageal squamous cell carcinoma and derived cell lines. Marked decreases in the level of miR-210 were observed especially in poorly differentiated carcinomas. We found that miR-210 inhibits cancer cell survival and proliferation by inducing cell death and cell cycle arrest in G_1/G_0 and G_2/M. Finally, we identified fibroblast growth factor receptor-like 1 (*FGFRL1*) as a target of miR-210 in esophageal squamous cell carcinoma and demonstrated that *FGFRL1* accelerates cancer cell proliferation by preventing cell cycle arrest in G_1/G_0. Taken together, our findings show an important role for miR-210 as a tumor-suppressive microRNA with effects on cancer cell proliferation.

MicroRNAs (miRNAs)[2] are evolutionarily conserved small noncoding RNAs (20–23 nucleotides) that bind to complementary sequences in the 3′-untranslated region (UTR) of target messenger RNAs (mRNAs) and regulate gene expression by the cleavage of target mRNAs and/or translational inhibition (1). Currently, >800 human miRNAs have been identified and registered in the miRNA database, miRBase (2). miRNAs play important roles in the differentiation of various cell types and in the initiation and progression of cancer, and it has been shown that the expression of some miRNAs is altered during cell differentiation and in malignancies (1, 3, 4).

In a recent study, we identified microRNA-210 (miR-210) as one of the miRNAs that is markedly differentially expressed during the process of epithelial differentiation (3). It has been reported that miR-210 expression is down-regulated during epithelial-mesenchymal transition, the aberrant activation of which triggers cancer pathology (5). Carcinomas are derived from epithelial cells, and poor prognosis in patients with carcinoma is associated with the disruption of characteristics of differentiated epithelial cells, such as cell junctions and polarity (6–8). Hence, given that the expression of miR-210 appears to be correlated well with epithelial differentiation, miR-210 might play a suppressive role in carcinomas. In support of this idea, allelic deletions at the miR-210 locus have been observed in 64% of cases of ovarian cancer (9), and ectopic expression of miR-210 represses tumor growth when human cancer cell lines are implanted into immunodeficient mice (10). However, the clinical roles of miR-210 in carcinomas and the mechanisms by which it represses tumor growth remain unknown.

In this study, we investigated the functional role of miR-210 in the growth of carcinomas and the mechanism by which it acts using clinical samples as well as cell lines of esophageal squamous cell carcinoma (ESCC). ESCC is a highly aggressive malignancy with a 5-year survival rate of 10% worldwide. It has been used as a model to study the mechanisms of dysregulated epithelial differentiation and epithelial-mesenchymal transition in carcinomas (11, 12).

EXPERIMENTAL PROCEDURES

Specimens—All tumor samples were confirmed as ESCC by the Clinicopathologic Department at Kyoto University Hospital. All cases were classified according to the sixth edition of the pathologic tumor-node-metastasis (TNM) classification (13). Written informed consent for the research use was obtained from each patient before surgery. The study was approved by the Kyoto University Institutional Review Board.

Cell Culture and Transfection—HE3 cells, which are human primary normal esophageal epithelial cells, were established and cultured in keratinocyte SFM (Invitrogen) containing human recombinant epidermal growth factor (Invitrogen) and bovine pituitary extract (Invitrogen). HEEpiC cells were purchased from ScienCell Research Laboratories (Carlsbad,

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3.
The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE20637).
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[2] The abbreviations used are: miRNA, microRNA; ESCC, esophageal squamous cell carcinoma; FGFRL1, fibroblast growth factor receptor-like 1; miR-210, microRNA-210; ncRNA, negative control RNA; ncsiRNA, negative control small interfering RNA; NEST, normal esophageal squamous tissue; PI, propidium iodide; qRT-PCR, quantitative reverse transcriptase-PCR.

CA) and were cultured according to the manufacturer's instructions (14). The human ESCC cell lines KYSE-150, -170, -190, and -590 were maintained as described previously (14, 15). KYSE-170 cells were transfected with oligoribonucleotides for miR-210 or negative control RNA (ncRNA) (Ambion, Austin, TX) using HiPerFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol for overexpression. KYSE-170 cells were also transfected with *FGFRL1* (fibroblast growth factor receptor-like 1) small interfering RNAs (siRNAs) or negative control siRNA (ncsiRNA) (Invitrogen) for knockdown of *FGFRL1*. The coding region of *FGFRL1* was isolated from complementary DNA (cDNA) from KTSE-170 cells, which was reverse transcribed using an oligo(dT) primer (Invitrogen), and ligated into the pcDNA3 expression vector. This *FGFRL1* expression construct was transfected into KYSE-170 cells using TransIT-LT1 transfection reagent (Mirus, Madison, WI). For inhibition of miRNAs function, KYSE-590 cells were transfected with a specific microRNA inhibitor for miR-210 (anti-miR-210) or its negative control RNA (anti-ncRNA) (Ambion) using HiPerFect transfection reagent.

RNA Extraction and Quantitative Reverse Transcriptase-PCR—Total RNA was extracted from the normal esophageal squamous tissues (NESTs), ESCCs, and cell lines by the acid guanidinium thiocyanate-phenol-chloroform method and then used for quantitative reverse transcriptase-PCR (qRT-PCR). To quantify mRNA expression levels, total RNA was reverse transcribed to cDNA using random primers and SuperScript II reverse transcriptase (Invitrogen), and quantitative PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was quantified using standard curves that were generated using serially diluted reference samples and normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The specificity of the PCR products was confirmed by gel electrophoresis and a dissociation curve analysis. Primer sequences are shown in supplemental Table 1. To quantify the levels of miRNAs and RNA U6 small nuclear 2 (*RNU6-2*), we used TaqMan MicroRNA assays (Applied Biosystems), which detect mature miRNAs specifically, following the manufacturer's protocol. The miRNA expression level was normalized to the expression level of *RNU6-2*.

Assay of Cell Proliferation, Cell Cycle, and Cell Death—The WST-1 assay to measure cell proliferation and flow cytometric assays to analyze the cell cycle and cell death were performed with KYSE-170 cells at 48 h after transfection as described previously (15). Calculations for the analysis of the cell cycle were performed with ModFit software (BD Biosciences). The bromodeoxyuridine (BrdU) incorporation assay, which measures cell proliferation, was performed using the BrdU Cell Proliferation ELISA kit (Roche Applied Science) according to the manufacturer's instructions.

Microarray Analysis—Total RNA from KYSE-170 cells that had been transfected with either ncRNA or miR-210 was labeled and prepared for hybridization to a human Oligo chip 25k (Toray, Tokyo, Japan) using standard methods. The GEO database accession code of the microarray data obtained is GSE20637.

Immunoblot Analyses—Cells were homogenized and centrifuged. The resultant supernatant was subjected to SDS-PAGE, and the separated proteins were transferred electrophoretically onto a PVDF membrane. An anti-FGFRL1, NDUFA4, GPR177, or LRP5L antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:100) was used as the primary antibody, respectively, and a horseradish peroxidase-conjugated IgG antibody (1:5,000) was used as the secondary antibody. The membranes were stained using an ECL kit according to the manufacturer's instructions. An anti-β-actin antibody (Sigma-Aldrich) (1:1,000) was used as a control.

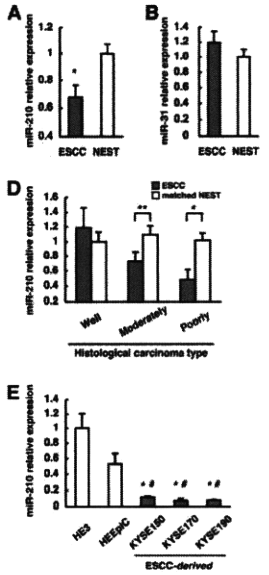
Immunostaining—Sections of ESCC and NEST were stained with an anti-FGFRL1 antibody (Santa Cruz Biotechnology) and ChemMate ENVISION kit (Dako, Glostrup, Denmark).

Luciferase Reporter Assay—The 3′-UTRs of *FGFRL1*, *WSB2*, and *GDI2* were isolated from cDNA from KTSE-170 cells. These 3′-UTRs and synthetic oligonucleotides of putative miR-210-target sites in the *FGFRL1* 3′-UTR ligated into the pGL3 basic luciferase expression (pGL3-Luc) vector (Promega, Madison, WI) at the 3′-end of the luciferase coding sequence (Luc), respectively. This pGL3-Luc vector containing 3′-UTR or miR-210 target sites and the pRL-TK internal control vector (Promega) were transfected into KYSE-170 cells using FuGENE HD transfection reagent (Roche Applied Science). Oligoribonucleotides for miR-210 or ncRNA were transfected 24 h after transfection of their vectors, and luciferase activity was measured 38 h after transfection of their vectors using the Dual Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions.

Statistics—Results are expressed as the mean ± S.E. Student's *t* test was used to compare data between two groups. *p* values less than 0.05 were considered to be statistically significant. In Figs. 1*C* and 4*E*, each clinicopathologic parameter, with the exception of age, was evaluated using Pearson's χ^2 test.

RESULTS

MiR-210 Expression Is Significantly Down-regulated in ESCC, Especially in Poorly Differentiated Carcinomas—To investigate the role of miR-210 in human malignancies, we first examined the expression levels of miR-210 in clinical samples of matched NEST and ESCC by qRT-PCR (Fig. 1*A*). Compared with NEST, a significant down-regulation of miR-210 expression was noted in ESCC. On the other hand, the expression of miR-31, which has been reported to be not altered between ESCC and NEST (16, 17), showed no significant difference (Fig. 1*B*). For further analysis, 82 clinical samples of ESCC were divided into two groups (miR-210-high and miR-210-low) on the basis of their miR-210 expression levels, and the clinicopathologic characteristics of these two groups were assessed. A significant difference was observed between the miR-210-high group and the miR-210-low group with respect to histological type (*i.e.* well, moderately, and poorly differentiated), but there were no significant differences between the two groups with respect to gender, age, or



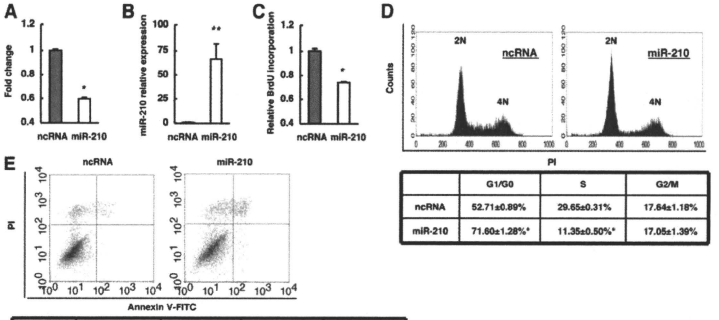
		miR-210-high	miR-210-low	*P*-value
Gender	Male	38	33	0.105
	Female	3	8	
Age	Mean	62.8	63.2	0.848
	Range	46 - 81	51 - 82	
TNM Stage	0	6	6	0.949
	I	5	4	
	IIa	6	6	
	IIb	15	15	
	III	2	4	
	IV	5	6	
Histological carcinoma type	Well differentiated	8	2	0.015
	Moderately differentiated	27	23	
	Poorly differentiated	6	16	

FIGURE 1. **Down-regulation of miR-210 expression in ESCC.** *A* and *B*, expression levels of miR-210 and miR-31 in NEST and ESCC assessed by qRT-PCR. The values are shown relative to the value obtained for NEST ($n = 82$; *, $p < 0.01$). *C*, clinicopathologic characteristics of 82 ESCCs divided into two groups ($n = 41$) on the basis of miR-210 expression levels. *D*, expression levels of miR-210 in the ESCCs and the corresponding NESTs in each histological type were compared by qRT-PCR. The values are shown relative to the value obtained for NEST in the well differentiated group (**, $p < 0.05$; *, $p < 0.01$). *E*, levels of miR-210 in HE3, HEEpiC, and KYSE cell lines analyzed by qRT-PCR. The values are shown relative to the value obtained for HE3 ($n = 3$; *, $p < 0.05$ *versus* HE3; #, $p < 0.05$ *versus* HEEpiC). *Error bars*, S.E.

pathologic TNM stage (Fig. 1*C*). Then, we compared the expression levels of miR-210 between ESCC and the matched NEST for each histological type. Significant differences were observed between moderately and poorly differentiated ESCC and the matched NEST (Fig. 1*D*). Moreover, as the degree of tumor differentiation decreased, the level of miR-210 showed a corresponding decrease in the ESCC ($p = 0.0875$, ANOVA) but not in the matched NEST ($p = 0.8728$, ANOVA), which indicated a strong correlation between the level of miR-210 and the degree of tumor differentiation in ESCC. This observation appears to agree well with the previous finding that miR-210 expression is up-regulated in parallel with epithelial cell differentiation (3, 18). Furthermore, when we compared the levels of miR-210 expression in normal human esophageal epithelial cells (HE3 and HEEpiC) with those in ESCC cell lines (KYSE-150, -170, and -190), we found that miR-210 expression was down-regulated significantly in the ESCC cell lines (Fig. 1*E*).

MiR-210 Inhibits Cancer Cell Proliferation by Inducing Cell Death and Cell Cycle Arrest in G_1/G_0 and G_2/M—Next, we examined the functional role of miR-210 in ESCC by adding synthetic miR-210 to the KYSE-170 cell line, in which miR-210 expression is low (Fig. 1*E*). Given that miR-210 expression was correlated with the level of differentiation in ESCC (Fig. 1, *C* and *D*), we examined the effect of miR-210 on the proliferation of ESCC cells. Transfection of miR-210 significantly decreased the proliferation of cancer cells, whereas a ncRNA had no inhibitory effect (Fig. 2*A*). Then, the introduced amount of miR-210 in cells transfected with miR-210 was a >60-fold increase compared with ones in cells transfected with ncRNA (Fig. 2*B*), which is within the comparable range for the differences between normal human esophageal epithelial cells and KYSE-170 cells (Fig. 1*E*). Moreover, we performed a BrdU incorporation assay and found that miR-210 significantly reduced the uptake of BrdU (Fig. 2*C*). These results suggested that miR-210 negatively regulates cancer cell proliferation. Next, we examined the effect of miR-210 on the cell cycle (Fig. 2*D*). Transfection of miR-210 resulted in a significant increase in the proportion of cells in G_1/G_0 phase as well as a significant decrease in the proportion of cells in S phase, whereas the proportion of cells in G_2/M phase was unaltered. These results indicate that miR-210 induces cell cycle arrest in both G_1/G_0 and G_2/M phases because cell cycle arrest only in G_1/G_0 phase decreases the proportion of cells in G_2/M phase. In addition, overexpression of miR-210 resulted in a significant increase in the proportion of FITC-annexin V-positive cells (Fig. 2*E*, *upper right* and *lower right*) and FITC-annexin V-negative/propidium iodide (PI)-positive cells (Fig. 2*E*, *upper left*), and a significant decrease in the proportion of FITC-annexin V-negative/PI-negative cells (Fig. 2*E*, *lower left*), which indicated that overexpression of miR-210 may lead to apoptosis and necrosis. Taken together, the results suggest that miR-210 may inhibit proliferation of ESCC cells mainly by inducing cell cycle arrest and apoptosis.

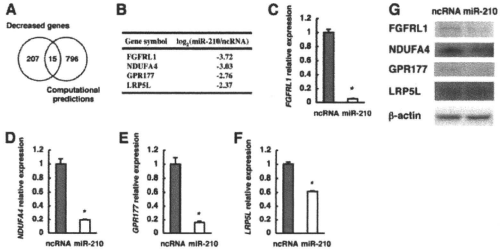
Identification of Candidate Target Genes Degraded by MiR-210—To investigate the molecular mechanism by which miR-210 inhibits ESCC cell proliferation, we analyzed the target genes of miR-210 in ESCC. We performed comprehen-



	G1/G0	S	G2/M
ncRNA	52.71±0.89%	29.65±0.31%	17.64±1.18%
miR-210	71.60±1.28%*	11.35±0.50%*	17.05±1.39%

	Upper Left	*Lower Left*	*Upper Right*	*Lower Right*
ncRNA	10.76±1.84%	83.47±1.68%	4.00±0.22%	1.77±0.15%
miR-210	15.28±1.58%**	74.45±2.00%*	7.03±0.78%*	3.25±0.15%*

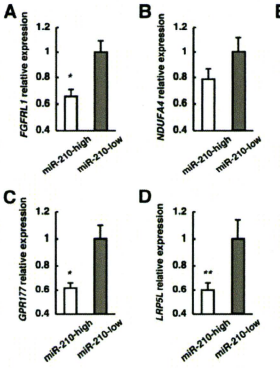
FIGURE 2. **Functions of miR-210 in KYSE-170 cells.** *A*, effect of miR-210 on cell proliferation was investigated by the WST-1 assay. Cells were incubated for 48 h after transfection with either ncRNA or miR-210, and viability was evaluated. The values are shown relative to the value obtained with ncRNA. *B*, expression levels of miR-210 in cells at 48 h after transfection with either ncRNA or miR-210 were investigated by qRT-PCR. The values are shown relative to the value obtained with ncRNA. *C*, 48 h after transfection, cell proliferation was evaluated by BrdU incorporation. The values are shown relative to the value obtained with ncRNA. *D*, effects of miR-210 on the cell cycle were investigated. The PI-stained DNA content of the cells was evaluated using a FACScan flow cytometer at 48 h after transfection. *E*, at 48 h after transfection, cells were stained with FITC-conjugated annexin V, and PI and cell death was evaluated using a FACScan flow cytometer. $n = 3$; **, $p < 0.05$; *, $p < 0.01$ *versus* ncRNA.



Gene symbol	$\log_2$(miR-210/ncRNA)
FGFRL1	-3.72
NDUFA4	-3.83
GPR177	-2.76
LRP5L	-2.37

FIGURE 3. **Identification of candidate target genes degraded by miR-210 in KYSE-170 cells.** *A*, Venn diagram showing overlapping sets of genes whose expression was decreased >5 fold by transfection of miR-210 and computationally predicted target genes of miR-210. *B*, list of four potential miR-210 target genes with a signal value of more than 50 in ncRNA-transfected cells upon microarray analysis. *C–F*, expression levels of the four potential miR-210 target mRNAs assessed by qRT-PCR. The values are shown relative to the value obtained with ncRNA ($n = 3$; *, $p < 0.01$). *G*, Western blot analyses of FGFRL1, NDUFA4, GPR177, LRP5L, and β-actin proteins.

sive transcriptome analysis using RNA from ESCC cells transfected with either ncRNA or miR-210. Assuming that the expression of target genes of miR-210 might be down-regulated in cells transfected with miR-210, we selected 222 genes whose expression was decreased by more than 5-fold in miR-210-transfected cells compared with ncRNA-transfected cells (supplemental Table 2). Fifteen of the 222 genes were predicted to be miR-210 target genes by the target prediction programs microcosm (2), TargetScan (19), and PicTar (20) (Fig. 3*A* and supplemental Tables 2 and 3). Four (*FGFRL1*, *NDUFA4*, *GPR177*, and *LRP5L*) of these 15 genes had a signal value of more than 50 in ncRNA-transfected cells (Fig. 3*B* and supplemental Table 2). Furthermore, their expression was confirmed by qRT-PCR and Western blotting; the mRNA expression of all four genes was significantly down-regulated by transfection of miR-210, although the degree of decrease of



		FGFRL1-high	FGFRL1-low	P-value
Gender	Male	35	36	0.746
	Female	6	5	
Age	Mean	63.6	62.4	0.547
	Range	51 - 81	46 - 82	
TNM Stage	0	8	4	0.687
	I	4	5	
	IIa	6	8	
	IIb	13	17	
	III	3	3	
	IV	7	4	
Histological carcinoma type	Well differentiated	2	8	0.037
	Moderately differentiated	24	26	
	Poorly differentiated	15	7	

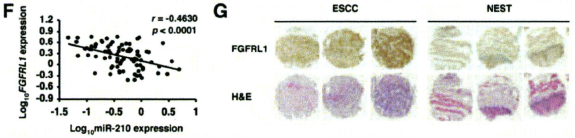


FIGURE 4. **Identification of candidate target genes of miR-210 in ESCC.** *A–D*, *FGFRL1*, *NDUFA4*, *GPR177*, and *LRP5L* expression levels in ESCCs divided into two groups on the basis of miR-210 expression levels were assessed by qRT-PCR. The values are shown relative to the value obtained for the miR-210-low group ($n = 41$; **, $p < 0.05$; *, $p < 0.01$). *E*, clinicopathologic characteristics of 82 ESCCs divided into two groups ($n = 41$) on the basis of *FGFRL1* expression levels. *F*, plot of $\log_{10}$*FGFRL1* relative expression intensity against $\log_{10}$miR-210 relative expression intensity. The *line* represents an approximated curve. The correlation coefficient (r) and the p value indicate the statistical significance of the negative correlation between the x and y variables. *G*, immunohistochemistry for FGFRL1 on ESCC and NEST. These sections were stained by anti-FGFRL1 antibody and by hematoxylin and eosin (*H&E*).

LRP5L was somewhat low (Fig. 3, *C–F*). The expression levels of FGFRL1, NDUFA4, and GPR177 proteins were also down-regulated by transfection of miR-210 (Fig. 3*G*). Meanwhile, the expression levels in LRP5L protein showed no change at 48 h after transfection of miR-210 (Fig. 3*G*). To validate these genes as targets of miR-210 further, we examined their expression in clinical ESCC samples. Of the four genes, the expression of *FGFRL1*, *GPR177*, and *LRP5L* was significantly down-regulated in the miR-210-high group compared with the miR-210-low group (Fig. 4, *A*, *C*, and *D*), whereas *NDUFA4* was not significantly different between the two groups (Fig. 4*B*). We then divided the 82 ESCC samples into two groups ($n = 41$) on the basis of their *FGFRL1*, *GPR177*, or *LRP5L* expression levels and compared the two groups in terms of their clinicopathologic characteristics. A significant association with histological type was observed for the *FGFRL1*-classified groups (Fig. 4*E*), but not for the *GPR177*- or *LRP5L*-classified ones (data not shown), which agrees well with the finding that a decrease in miR-210 expression correlates well with the poorly differentiated state of ESCC. A statistically significant negative correlation was obtained between expression levels of miR-210 and that of *FGFRL1* in ESCC (Fig. 4*F*). Furthermore, sections of ESCC and NEST were stained by immunohistochemistry for FGFRL1. The results indicated that FGFRL1 protein is expressed in ESCC and the expression levels are enhanced compared with NEST (Fig. 4*G*).

MiR-210 Targets FGFRL1 3′-UTR Directly—Five sites in the *FGFRL1* 3′-UTR are predicted to be potential target sites of miR-210 by microcosm (Fig. 5*A*), although none of them is conserved across species such as mouse, rat, and chicken. To examine whether *FGFRL1* is a direct target of miR-210, we cloned the 3′-UTR of *FGFRL1* into a pGL3-Luc vector (pGL3-Luc-*FGFRL1* 3′-UTR) to perform reporter assay. When miR-210 was transfected into the cells with this reporter construct but not the mock, luciferase activity was repressed more than 50% compared with transfection of ncRNA (Fig. 5*B*). Meanwhile, luciferase activities in cells transfected with reporter constructs containing the 3′-UTR of *WSB2 or GDI2* were not repressed by the transfection of miR-210 (Fig. 5*B*). *WSB2* and *GDI2* were not included in either predicted target genes of miR-210 or 222 genes decreased in miR-210-transfected cells (supplemental Tables 2 and 3). These results suggest that

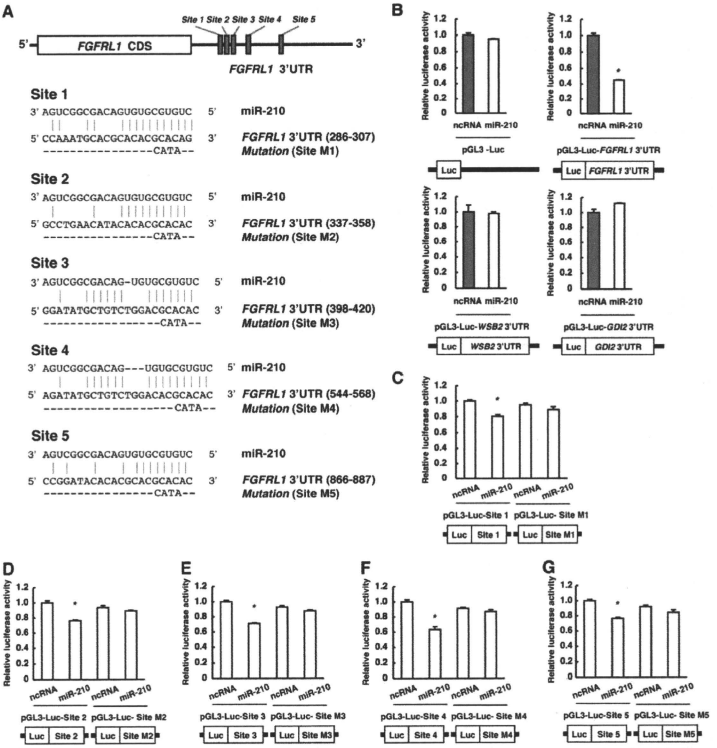
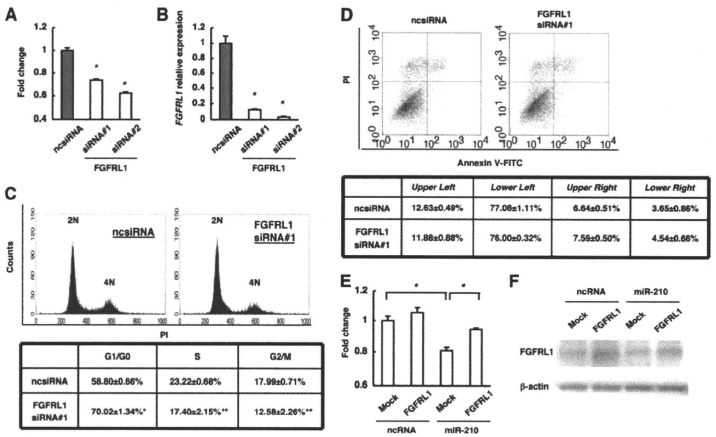


FIGURE 5. **Identification of miR-210 target sites in *FGFRL1* 3′-UTR.** *A*, schematic diagram of potential miR-210-target sites in *FGFRL1* 3′-UTR. *B–G*, cells were transfected with either ncRNA or miR-210 at 24 h after transfection with the pGL3 luciferase expression vector containing 3′-UTR of each gene or candidate miR-210 target site in *FGFRL1* 3′-UTR. After 14 h, reporter luciferase activity was evaluated. The values are shown relative to the value obtained with ncRNA ($n = 3$; *, $p < 0.01$).

FGFRL1 is a direct and robust target gene of miR-210 in ESCC. For further analysis, we cloned the each putative miR-210-target site or its point mutant in sequences corresponded to "seed sequence" of miR-210 into a pGL3-Luc vector (Fig. 5*A*) and performed reporter assays (Fig. 5, *C–G*). When miR-210 was transfected into the cells, luciferase activity was significantly repressed in all five sites. By contrast, when mutation was introduced in these sites, the repressions by miR-210 were completely abolished in all cases (Fig. 5, *C–G*). These results suggested that these five sites in the *FGFRL1* 3′-UTR are target sites of miR-210.

FGFRL1 Accelerates Cancer Cell Proliferation by Preventing Cell Cycle Arrest in G_1/G_0—To investigate the roles of *FGFRL1* in cancer cell proliferation, we first examined the effect of *FGFRL1* knockdown by using two types of *FGFRL1* siRNA. With both siRNAs, knockdown of *FGFRL1* significantly de-



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ncsiRNA	12.63±0.49%	77.08±1.11%	6.64±0.51%	3.65±0.86%
FGFRL1 siRNA#1	11.88±0.88%	76.00±0.32%	7.59±0.50%	4.54±0.66%

	G1/G0	S	G2/M
ncsiRNA	58.80±0.66%	23.22±0.68%	17.99±0.71%
FGFRL1 siRNA#1	70.02±1.34%*	17.40±2.15%**	12.58±2.26%**

FIGURE 6. **Functions of FGFRL1 in KYSE-170 cells.** *A*, effect of *FGFRL1* on cell proliferation was investigated by the WST-1 assay. Cells were incubated for 48 h after transfection with ncsiRNA, *FGFRL1* siRNA1 or 2, and viability was evaluated. The values are shown relative to the value obtained with ncsiRNA (n = 3; *, $p < 0.01$ *versus* ncsiRNA). *B*, *FGFRL1* expression levels in *A* were assessed by qRT-PCR. The values are shown relative to the value obtained with ncsiRNA (n = 3; *, $p < 0.01$ *versus* ncsiRNA). *C*, effects of *FGFRL1* on the cell cycle were investigated. The PI-stained DNA content of the cells was evaluated using a FACScan flow cytometer at 48 h after transfection (n = 3; **, $p < 0.05$; *, $p < 0.01$ *versus* ncsiRNA). *D*, at 48 h after transfection, cells were stained with FITC-conjugated annexin V and PI, and cell death was evaluated using a FACScan flow cytometer (n = 3). *E*, cells were transfected with either ncRNA or miR-210 at 24 h after transfection with the *FGFRL1* expression vector or mock. After 48 h, viability was evaluated. The values are shown relative to the value obtained with ncRNA and mock transfection (n = 3; *, $p < 0.01$). *F*, Western blot analyses of FGFRL1 and β-actin proteins in *E* are shown.

creased proliferation of ESCC cells, whereas a ncsiRNA had no inhibitory effect (Fig. 6*A*). The level of *FGFRL1* expression was reduced to ~10% of the original level with both siRNAs (Fig. 6*B*). Next, we examined the effects of *FGFRL1* knockdown on the cell cycle (Fig. 6*C*). Knockdown of *FGFRL1* resulted in a significant increase in the proportion of cells in G_1/G_0 phase and a decrease in that in S and G_2/M phases. These results indicated that down-regulation of *FGFRL1* induced cell cycle arrest in G_1/G_0. Down-regulation of *FGFRL1* did not significantly change the proportions of FITC-annexin V-positive cells, FITC-annexin V-negative/PI-positive cells, and FITC-annexin V-negative/PI-negative cells, which indicated that *FGFRL1* is not involved in apoptosis and necrosis (Fig. 6*D*). In contrast, overexpression of *FGFRL1* with no 3′-UTR significantly reduced the inhibitory effect of miR-210 on cell proliferation (Fig. 6*E*). Then, expression levels of FGFRL1 proteins are actually increased in cells transfected with a FGFRL1 expression vector without the 3′-UTR of *FGFRL1* (Fig. 6*F*). These findings suggest that *FGFRL1* increases the proliferation of ESCC cells by inhibiting cell cycle arrest in G_1/G_0 phase.

Endogenous MiR-210 Regulates Expression Levels of FGFRL1 and Cancer Cell Proliferation—To examine whether endogenous miR-210 regulates the expression levels of endogenous *FGFRL1*, we examined the effect of anti-miR-210 in KYSE-590 cells, which relatively highly expresses miR-210. The treatment by 2′-*O*-methylated antisense RNA of miR-210 significantly enhanced the expression levels of FGFRL1 mRNA and protein (Fig. 7*A*). Then, cancer cell proliferation was significantly increased (Fig. 7*B*). These results may indicate that miR-210 endogenously regulates the expression levels of *FGFRL1* and cancer cell proliferation.

DISCUSSION

In the present study, we showed for the first time that expression of miR-210 is down-regulated in ESCC cell lines as well as in clinical samples and that miR-210 induces cell cycle arrest and apoptosis *in vitro*, thus inhibiting the proliferation of cancer cells. Furthermore, we found that not only miR-210 expression was decreased in ESCC, but the degree of tumor differentiation corresponded well with the levels of miR-210 in clinical ESCC samples; thus, more poorly differentiated ESCCs exhibited less miR-210 expression. The results indicate that the level of miR-210 expression may serve as a clinical maker for the degree of tumor differentiation *in vivo*. We also examined whether the lower levels of miR-210 expression in poorly differentiated ESCC were related to the enhanced

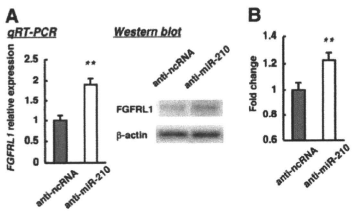

FIGURE 7. **Effects of functional inhibition of endogenous miR-210.** *A*, effect of inhibition of endogenous miR-210 on *FGFRL1* expression levels was assessed by qRT-PCR and Western blot analyses using KYSE-590 cells. Cells were incubated for 48 h after transfection with anti-ncRNA or anti-miR-210. The values are shown relative to the value obtained with anti-ncRNA ($n = 3$; **, $p < 0.05$ *versus* anti-ncRNA). *B*, cell viability in *A* was evaluated by the WST-1 assay. The values are shown relative to the value obtained with anti-ncRNA ($n = 3$; **, $p < 0.05$ *versus* anti-ncRNA).

proliferation of such carcinomas, and we found that miR-210 acts as a tumor-suppressive miRNA in ESCC.

In contrast to our study, miR-210 was reported to be up-regulated in breast cancer, pancreatic tumors, and head and neck cancer and to be correlated with their poor outcome (21–24). Moreover, previous studies have shown that miR-210 inhibits apoptosis and bypasses cell cycle arrest in cancer cell lines such as MCF-7 and HCT116 Dicer^{ex5} cells (25, 26). Thus, our finding appears to be different from the previous reports that miR-210 is up-regulated in cancer tissues and enhances cell survival. The reason for this apparent discrepancy is uncertain. A likely explanation in the function of miR-210 is that it is possible that the expression of miR-210 might only reflect the hypoxia status in these cases as a surrogate marker for tumor hypoxia because miR-210 is the most robustly induced miRNA under hypoxia (9, 25, 26). Additionally, the apparently discrepant results in cancer cell lines might be explained by cell type-specific differences in the expression levels of target genes of miR-210. The results of our transcriptome analysis on ESCC cells that expressed miR-210 ectopically supported this possibility because induction of miR-210 did not affect the expressions of *CASP8AP2* and *MNT* (supplemental Table 2), which are target genes of miR-210 and induce apoptosis and cell cycle arrest, respectively (26, 27). Similar to our findings, Huang *et al.* have reported recently that miR-210 represses the initiation of tumor growth when human head and neck or pancreatic tumor cell lines that are expressing miR-210 ectopically are implanted into immunodeficient mice (10).

In the present study, we found that miR-210 is targeted to the *FGFRL1* 3′-UTR and suppresses *FGFRL1* expression in ESCC. Five sites in the 3′-UTR of *FGFRL1* were identified as target sites of miR-210. The repression in the *FGFRL1* 3′-UTR containing these five sites was considerably larger than that in each miR-210 target sites. These five miR-210 target sites in the *FGFRL1* 3′-UTR may function synergistically in the decrease in the *FGFRL1* expression level. It has been actually reported that closely located multiple miRNA target sites in the 3′-UTR of a gene may function synergistically (28, 29). As suggested by the observation that overexpression of miR-210 in ESCC cells down-regulated expression of *FGFRL1* more than that of the other predicted target genes, we found that *FGFRL1* mRNA expression was down-regulated in the miR-210-high group of clinical samples and that the level of expression was correlated inversely with the degree of differentiation. Moreover, knockdown of *FGFRL1* by siRNA inhibited ESCC cell proliferation, whereas overexpression of *FGFRL1* effectively rescued the miR-210-induced suppression of ESCC cell proliferation. Together, these findings show that miR-210 might exert its tumor-suppressive effect in ESCC mainly by targeting *FGFRL1*. This conclusion was supported by a recent study showing that *FGFRL1* could partially rescue the phenotype (suppression of tumor growth) caused by ectopic expression of miR-210 in tumor xenografts (10). However, targeting of *FGFRL1* by miR-210 appears to explain only part of the action of miR-210: down-regulation of *FGFRL1* arrested the cell cycle in G_1/G_0 phase, but not in G_2/M phase, although miR-210 induced cell cycle arrest in both phases. Hence, miR-210-induced cell cycle arrest in G_2/M phase and apoptosis might be regulated by other targets of miR-210. In fact, our transcriptome analysis identified several genes whose expression was regulated differentially by miR-210. Further studies are clearly required to understand fully the molecular mechanism of tumor suppression by miR-210.

In conclusion, the results of our present study show that miR-210 inhibits the proliferation of ESCC cells by inducing cell cycle arrest and apoptosis and that the effects of miR-210 are mediated mainly by the targeting of *FGFRL1*. Our data suggest that down-regulation of miR-210 might play an important role in the proliferation of ESCC and that miR-210 and *FGFRL1* might serve as clinical markers for tumor differentiation and therapeutic targets of ESCC.

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Supplementary Table 1. List of primers used for real-time PCR analysis

Gene Symbol	Forward (5' -> 3')	Reverse (5' -> 3')
FGFRL1	acacagccctccaagatgag	gcaggttcttcaggctcagt
NDUFA4	agcttgatccccctctttgt	ctggacgttccttcttcagc
GPR177	aggcatctatgggatgtgga	ggaatatttcgaagcgctga
LRP5L	ctcaaagctgtgaacgtgga	gcggctctactggtgaagac
GAPDH	gagtcaacggatttggtcgt	ttgattttggagggatctcg

Supplementary Table 2. The list represents 222 genes decreased by more than five-fold in miR-210-tansfected cells compared with ncRNA-tansfected cells

Gene Symbol	description	ncRNA	miR-210	Log.(ratio)
IGHG3	Immunoglobulin heavy chain C gene segment [Source:IMGT/GENE-DB;Acc:IGHG3]	41	1	-5.36
CYB5R3	NADH-cytochrome b5 reductase (EC 1.6.2.2) (B5R) (Diaphorase-1) (Cytochrome b5 reductase 3) [Contains: NADH-cytochrome b5 reductase membrane-bound form; NADH-cytochrome b5 reductase soluble form]. [Source:Uniprot/SWISSPROT;Acc:P00387]	40	1	-5.32
FAM19A4	[Source:Uniprot/SWISSPROT;Acc:Q96LR4]	39	1	-5.29
COBL	Protein cordon-bleu. [Source:Uniprot/SWISSPROT;Acc:O75128]	37	1	-5.21
BST2	Bone marrow stromal antigen 2 precursor (BST-2) (CD317 antigen) (HM1.24 antigen). [Source:Uniprot/SWISSPROT;Acc:Q10589]	35	1	-5.13
ONCO_HUMAN	Oncomodulin (OM) (Parvalbumin beta). [Source:Uniprot/SWISSPROT;Acc:P32930]	35	1	-5.13
POU3F1	POU domain, class 3, transcription factor 1 (Octamer-binding transcription factor 6) (Oct-6) (POU domain transcription factor SCIP). [Source:Uniprot/SWISSPROT;Acc:Q03052]	35	1	-5.13
PTPRT	Receptor-type tyrosine-protein phosphatase T precursor (EC 3.1.3.48) (R-PTP-T) (RPTP-rho). [Source:Uniprot/SWISSPROT;Acc:O14522]	34	1	-5.09
PROM1	Prominin-1 precursor (Prominin-like protein 1) (Antigen AC133) (CD133 antigen). [Source:Uniprot/SWISSPROT;Acc:O43490]	33	1	-5.04
ARTS1_HUMAN	(Aminopeptidase PILS) (Puromycin-insensitive leucyl- specific aminopeptidase) (PILS-AP) (Type 1 tumor necrosis factor receptor shedding aminopeptidase regulator). [Source:Uniprot/SWISSPROT;Acc:Q9NZ08]	29	1	-4.86
Q96I54_HUMAN	-	28	1	-4.81
CALN1	Calneuron-1 (Calcium-binding protein CaBP8). [Source:Uniprot/SWISSPROT;Acc:Q9BXU9]	28	1	-4.81
UGT2B4	UDP-glucuronosyltransferase 2B4 precursor (EC 2.4.1.17) (UDPGT) (Hydroxycholic acid) (HLUG25) (UDPGTh-1). [Source:Uniprot/SWISSPROT;Acc:P06133]	27	1	-4.75
GABRB1	Gamma-aminobutyric-acid receptor subunit beta-1 precursor (GABA(A) receptor subunit beta-1). [Source:Uniprot/SWISSPROT;Acc:P18505]	26	1	-4.7
DFFB	DNA fragmentation factor subunit beta (EC 3.-.-.-) (DNA fragmentation factor 40 kDa subunit) (DFF-40) (Caspase-activated deoxyribonuclease) (Caspase-activated DNase) (CAD) (Caspase-activated nuclease) (CPAN). [Source:Uniprot/SWISSPROT;Acc:O76075]	26	1	-4.7
GABRD	Gamma-aminobutyric-acid receptor subunit delta precursor (GABA(A) receptor subunit delta). [Source:Uniprot/SWISSPROT;Acc:O14764]	26	1	-4.7
Q5JV89_HUMAN	-	25	1	-4.64
MANEAL	mannosidase, endo-alpha-like isoform 2 [Source:RefSeq_peptide;Acc:NP_689709]	25	1	-4.64
MEGF6	Multiple epidermal growth factor-like domains 6 precursor (EGF-like domain-containing protein 3) (Multiple EGF-like domain protein 3). [Source:Uniprot/SWISSPROT;Acc:O75095]	24	1	-4.58
TIMP4	Metalloproteinase inhibitor 4 precursor (TIMP-4) (Tissue inhibitor of metalloproteinases 4). [Source:Uniprot/SWISSPROT;Acc:Q99727]	23	1	-4.52
TNFAIP6	Tumor necrosis factor-inducible protein TSG-6 precursor (TNF- stimulated gene 6 protein) (Hyaluronate-binding protein). [Source:Uniprot/SWISSPROT;Acc:P98066]	23	1	-4.52
IL24	Interleukin-24 precursor (Suppression of tumorigenicity 16 protein) (Melanoma differentiation-associated gene 7 protein) (MDA-7). [Source:Uniprot/SWISSPROT;Acc:Q13007]	23	1	-4.52
Q8NHA6_HUMAN	Seven transmembrane helix receptor. [Source:Uniprot/SPTREMBL;Acc:Q8NHA6]	23	1	-4.52
PDE1B	Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B (EC 3.1.4.17) (Cam-PDE 1B) (63 kDa Cam-PDE). [Source:Uniprot/SWISSPROT;Acc:Q01064]	23	1	-4.52
EHBP1L1	Signal-induced proliferation-associated protein 1 (Sipa-1) (GTPase- activating protein Spa-1) (p130 SPA-1). [Source:Uniprot/SWISSPROT;Acc:Q96FS4]	22	1	-4.46
C14orf174	C14orf174 protein. [Source:Uniprot/SPTREMBL;Acc:Q2M3P3]	43	2	-4.43
PLEKHG1	[Source:Uniprot/SWISSPROT;Acc:Q9ULL1]	21	1	-4.39
ASTN1	Astrotactin-1 precursor. [Source:Uniprot/SWISSPROT;Acc:O14525]	20	1	-4.32
PSG1	Pregnancy-specific beta-1-glycoprotein 1 precursor (PSBG-1) (Pregnancy-specific beta-1 glycoprotein C/D) (PS-beta-C/D) (Fetal liver non-specific cross-reactive antigen 1/2) (FL-NCA-1/2) (PSG95) (CD66f antigen). [Source:Uniprot/SWISSPROT;Acc:P11464]	20	1	-4.32
SLC6A20	Sodium- and chloride-dependent transporter XTRP3 (Solute carrier family 6 member 20) (Neurotransmitter transporter rB21A homolog). [Source:Uniprot/SWISSPROT;Acc:Q9NP91]	20	1	-4.32
LRRN1	Leucine-rich repeat neuronal protein 1 precursor (Neuronal leucine- rich repeat protein 1) (NLRR-1). [Source:Uniprot/SWISSPROT;Acc:Q6UXK5]	39	2	-4.29
IFT57	estrogen-related receptor beta like 1 [Source:RefSeq_peptide;Acc:NP_060480]	19	1	-4.25
Q96LL3_HUMAN	CDNA FLJ25404 fis, clone TST02888 (Hypothetical protein FLJ25404). [Source:Uniprot/SPTREMBL;Acc:Q96LL3]	19	1	-4.25
COCH	Cochlin precursor (COCH-5B2). [Source:Uniprot/SWISSPROT;Acc:O43405]	19	1	-4.25
NP_683701.2	GREB1 protein isoform b [Source:RefSeq_peptide;Acc:NP_149081]	19	1	-4.25
Q8NAW6_HUMAN	CDNA FLJ34651 fis, clone KIDNE2018167. [Source:Uniprot/SPTREMBL;Acc:Q8NAW6]	19	1	-4.25
TAF6L	TAF6-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 6L (PCAF-associated factor 65 alpha) (PAF65- alpha). [Source:Uniprot/SWISSPROT;Acc:Q9Y6J9]	18	1	-4.17
TMPRSS12	transmembrane protease, serine 12 [Source:RefSeq_peptide;Acc:NP_872365]	18	1	-4.17
Q8NAT4_HUMAN	CDNA FLJ34815 fis, clone NT2NE2007786. [Source:Uniprot/SPTREMBL;Acc:Q8NAT4]	17	1	-4.09
SLC12A3	Solute carrier family 12 member 3 (Thiazide-sensitive sodium-chloride cotransporter) (Na-Cl symporter). [Source:Uniprot/SWISSPROT;Acc:P55017]	17	1	-4.09
CDON	Cell adhesion molecule-related/down-regulated by oncogenes precursor. [Source:Uniprot/SWISSPROT;Acc:Q4KMG0]	17	1	-4.09
SGCG	Gamma-sarcoglycan (Gamma-SG) (35 kDa dystrophin-associated glycoprotein) (35DAG). [Source:Uniprot/SWISSPROT;Acc:Q13326]	17	1	-4.09
DCDC2	[Source:Uniprot/SWISSPROT;Acc:Q9UHG0]	17	1	-4.09

Supplementary Table 2. The list represents 222 genes decreased by more than five-fold in miR-210-tansfected cells compared with ncRNA-tansfected cells

Gene Symbol	description	ncRNA	miR-210	Log_2(ratio)
PTF1A	Pancreas transcription factor 1 subunit alpha (Pancreas-specific transcription factor 1a) (bHLH transcription factor p48) (p48 DNA- binding subunit of transcription factor PTF1) (PTF1-p48). [Source:Uniprot/SWISSPROT;Acc:Q7RTS3]	34	2	-4.09
IGSF5	IGSF5 protein (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q9NSI5]	17	1	-4.09
TECTA	Alpha-tectorin precursor. [Source:Uniprot/SWISSPROT;Acc:O75443]	17	1	-4.09
LRTM2	Leucine-rich repeat and transmembrane domain-containing protein 2 precursor. [Source:Uniprot/SWISSPROT;Acc:Q8N967]	16	1	-4
MYT1L	Myelin transcription factor 1-like protein (MyT1L protein) (MyT1-L). [Source:Uniprot/SWISSPROT;Acc:Q9UL68]	16	1	-4
Q9H5H6_HUMAN	CDNA: FLJ23429 fis, clone HRC10578. [Source:Uniprot/SPTREMBL;Acc:Q9H5H6]	16	1	-4
LIX1	Protein limb expression 1 homolog. [Source:Uniprot/SWISSPROT;Acc:Q8N485]	32	2	-4
FSTL5	Follistatin-related protein 5 precursor (Follistatin-like 5). [Source:Uniprot/SWISSPROT;Acc:Q8N475]	16	1	-4
SEMG2	Semenogelin-2 precursor (Semenogelin II) (SGII). [Source:Uniprot/SWISSPROT;Acc:Q02383]	15	1	-3.91
Q16653-6	[Source:RefSeq_peptide;Acc:NP_996536]	15	1	-3.91
Q4VXH5_HUMAN	XAGE-4 protein (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q8WWM0]	15	1	-3.91
SERPINA2	Alpha-1-antitrypsin-related protein precursor. [Source:Uniprot/SWISSPROT;Acc:P20848]	15	1	-3.91
GSDMDC1	Gasdermin domain-containing protein 1. [Source:Uniprot/SWISSPROT;Acc:P57764]	15	1	-3.91
Q8TBU5_HUMAN	-	14	1	-3.81
ZNF83	Zinc finger protein 83 (Zinc finger protein HPF1). [Source:Uniprot/SWISSPROT;Acc:P51522]	14	1	-3.81
REG1A	Lithostathine 1 alpha precursor (Pancreatic stone protein) (PSP) (Pancreatic thread protein) (PTP) (Islet of Langerhans regenerating protein) (REG) (Regenerating protein I alpha) (Islet cells regeneration factor) (ICRF). [Source:Uniprot/SWISSPROT;Acc:P05451]	14	1	-3.81
GRIP1	[Source:Uniprot/SWISSPROT;Acc:Q9Y3R0]	28	2	-3.81
FGFRL1	Fibroblast growth factor receptor-like 1 precursor (FGF receptor-like protein 1) (Fibroblast growth factor receptor 5) (FGFR-like protein) (FGF homologous factor receptor). [Source:Uniprot/SWISSPROT;Acc:Q8N441]	277	21	-3.72
SHBG	Sex hormone-binding globulin precursor (SHBG) (Sex steroid-binding protein) (SBP) (Testis-specific androgen-binding protein) (ABP) (Testosterone-estrogen-binding globulin) (Testosterone-estradiol-binding globulin) (TeBG). [Source:Uniprot/SWISSPROT;Acc:P04278]	13	1	-3.7
PPIL2	Peptidyl-prolyl cis-trans isomerase-like 2 (EC 5.2.1.8) (PPIase) (Rotamase) (Cyclophilin-60) (Cyclophilin-like protein Cyp-60). [Source:Uniprot/SWISSPROT;Acc:Q13356]	13	1	-3.7
IGSF21	Immunoglobulin superfamily member 21 precursor. [Source:Uniprot/SWISSPROT;Acc:Q96ID5]	26	2	-3.7
IL21	Interleukin-21 precursor (IL-21) (Za11). [Source:Uniprot/SWISSPROT;Acc:Q9HBE4]	13	1	-3.7
CLIC6	Chloride intracellular channel 6. [Source:Uniprot/SWISSPROT;Acc:Q96NY7]	13	1	-3.7
SLITRK2	SLIT and NTRK-like protein 2 precursor. [Source:Uniprot/SWISSPROT;Acc:Q9H156]	12	1	-3.58
RIMS1	Regulating synaptic membrane exocytosis protein 1 (Rab3-interacting molecule 1) (RIM 1). [Source:Uniprot/SWISSPROT;Acc:Q86UR5]	12	1	-3.58
LAYN	Layilin precursor. [Source:Uniprot/SWISSPROT;Acc:Q6UX15]	12	1	-3.58
ASB17	Ankyrin repeat and SOCS box protein 17 (ASB-17). [Source:Uniprot/SWISSPROT;Acc:Q8WXJ9]	12	1	-3.58
RAD21L1	Syntaphilin. [Source:Uniprot/SWISSPROT;Acc:O15079]	12	1	-3.58
FGF23	Fibroblast growth factor 23 precursor (FGF-23) (Tumor-derived hypophosphatemia-inducing factor). [Source:Uniprot/SWISSPROT;Acc:Q9GZV9]	12	1	-3.58
CAMTA1	Calmodulin-binding transcription activator 1. [Source:Uniprot/SWISSPROT;Acc:Q9Y6Y1]	23	2	-3.52
APOA2	Apolipoprotein A-II precursor (Apo-AII) (ApoA-II) [Contains: Apolipoprotein A-II(1-76)]. [Source:Uniprot/SWISSPROT;Acc:P02652]	11	1	-3.46
NP_861450.1	LOC283537 protein (OTTHUMP00000018184). [Source:Uniprot/SPTREMBL;Acc:Q6P9B3]	11	1	-3.46
NDUFA5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 (EC 1.6.5.3) (EC 1.6.99.3) (NADH-ubiquinone oxidoreductase 13 kDa-B subunit) (Complex I-13kD-B) (CI-13kD-B) (Complex I subunit B13). [Source:Uniprot/SWISSPROT;Acc:Q16718]	11	1	-3.46
DDO	D-aspartate oxidase (EC 1.4.3.1) (DASOX) (DDO). [Source:Uniprot/SWISSPROT;Acc:Q99489]	22	2	-3.46
BCORL2	BCoR-like protein 2 (BCL-6 corepressor-like protein 2). [Source:Uniprot/SWISSPROT;Acc:Q8N888]	11	1	-3.46
NM_207504	CDNA FLJ46365 fis, clone TESTI4051054. [Source:Uniprot/SPTREMBL;Acc:Q6ZRG8]	11	1	-3.46
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	22	2	-3.46
CDKN1C	Cyclin-dependent kinase inhibitor 1C (Cyclin-dependent kinase inhibitor p57) (p57KIP2). [Source:Uniprot/SWISSPROT;Acc:P49918]	11	1	-3.46
DNALI1	Axonemal dynein light intermediate polypeptide 1 (Inner dynein arm light chain, axonemal) (hp28). [Source:Uniprot/SWISSPROT;Acc:Q14645]	11	1	-3.46
MCHR2	(MCH-2R) (MCH2) (G-protein coupled receptor 145) (GPRv17). [Source:Uniprot/SWISSPROT;Acc:Q969V1]	21	2	-3.39
DNAH2	dynein heavy chain domain 3 [Source:RefSeq_peptide;Acc:NP_065928]	21	2	-3.39
RTN2	Reticulon-2 (Neuroendocrine-specific protein-like 1) (NSP-like protein 1) (NSPLI). [Source:Uniprot/SWISSPROT;Acc:O75298]	21	2	-3.39
ASIP	[Source:Uniprot/SWISSPROT;Acc:P42127]	10	1	-3.32
AMPH	Amphiphysin. [Source:Uniprot/SWISSPROT;Acc:P49418]	10	1	-3.32
FBXL21	F-box and leucine-rich repeat protein 21 [Source:RefSeq_peptide;Acc:NP_036291]	10	1	-3.32
ANGPTL2	[Source:Uniprot/SWISSPROT;Acc:Q9UKU9]	30	3	-3.32
Q96RZ4_HUMAN	-	30	3	-3.32
C12orf50	C12orf50 protein (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q6P674]	20	2	-3.32

Supplementary Table 2. The list represents 222 genes decreased by more than five-fold in miR-210-tansfected cells compared with ncRNA-tansfected cells

Gene Symbol	description	ncRNA	miR-210	Log_2(ratio)
MSMB	Beta-microseminoprotein precursor (Prostate secreted seminal plasma protein) (Prostate secretory protein PSP94) (PSP-94) (Seminal plasma beta-inhibin) (Immunoglobulin-binding factor) (IGBF) (PN44). [Source:Uniprot/SWISSPROT;Acc:P08118]	20	2	-3.32
HKR2_HUMAN	Krueppel-related zinc finger protein 2 (Protein HKR2) (Zinc finger protein 50) (Zinc finger and SCAN domain-containing protein 22) (Fragment). [Source:Uniprot/SWISSPROT;Acc:P10073]	10	1	-3.32
HIVEP3	[Source:RefSeq_peptide;Acc:NP_078779]	19	2	-3.25
GPR35	Probable G-protein coupled receptor 35. [Source:Uniprot/SWISSPROT;Acc:Q9HC97]	19	2	-3.25
C1orf145	C1orf145 protein (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q8N372]	19	2	-3.25
CBX6	Chromobox protein homolog 6. [Source:Uniprot/SWISSPROT;Acc:O95503]	28	3	-3.22
Q9H353_HUMAN	-	37	4	-3.21
C11orf69	Uncharacterized protein C11orf69. [Source:Uniprot/SWISSPROT;Acc:Q8TAY4]	37	4	-3.21
MYF5	Myogenic factor 5 (Myf-5). [Source:Uniprot/SWISSPROT;Acc:P13349]	9	1	-3.17
PRKAR2B	[Source:Uniprot/SWISSPROT;Acc:P31323]	9	1	-3.17
NOX1	NADPH oxidase homolog 1 (NOX-1) (NOH-1) (NADH/NADPH mitogenic oxidase subunit P65-MOX) (Mitogenic oxidase 1) (MOX1). [Source:Uniprot/SWISSPROT;Acc:Q9Y5S8]	9	1	-3.17
ELOVL3	Elongation of very long chain fatty acids protein 3 (Cold-inducible glycoprotein of 30 kDa). [Source:Uniprot/SWISSPROT;Acc:Q9HB03]	45	5	-3.17
BCL2L10	Apoptosis regulator Bcl-B (Bcl-2-like 10 protein) (Bcl2-L-10) (Anti- apoptotic protein NrH). [Source:Uniprot/SWISSPROT;Acc:Q9HD36]	18	2	-3.17
SEPT8	Septin-8. [Source:Uniprot/SWISSPROT;Acc:Q92599]	18	2	-3.17
C17orf57	C17orf57 protein. [Source:Uniprot/SPTREMBL;Acc:Q49AG9]	18	2	-3.17
SPANXN2	SPANX-N2 protein [Source:RefSeq_peptide;Acc:NP_001009615]	9	1	-3.17
CHST5	(GlcNAc6ST-3) (Intestinal GlcNAc-6- sulfotransferase) (Intestinal N-acetylglucosamine-6-O-sulfotransferase) (I-GlcNAc6ST) (hIGn6ST) (Galactose/N- acetylglucosamine [Source:Uniprot/SWISSPROT;Acc:Q9GZS9]	26	3	-3.12
DUOXA2	[Source:Uniprot/SWISSPROT;Acc:Q1HG44]	26	3	-3.12
FAM26A	Protein FAM26A. [Source:Uniprot/SWISSPROT;Acc:Q86XJ0]	69	8	-3.11
NEURL2	Neuralized-like protein 2. [Source:Uniprot/SWISSPROT;Acc:Q9BR09]	43	5	-3.1
TERT	Telomerase reverse transcriptase (EC 2.7.7.49) (Telomerase catalytic subunit) (HEST2) (Telomerase-associated protein 2) (TP2). [Source:Uniprot/SWISSPROT;Acc:O14746]	34	4	-3.09
NDUFA4	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4 (EC 1.6.5.3) (EC 1.6.99.3) (NADH-ubiquinone oxidoreductase MLRQ subunit) (Complex I-MLRQ) (CI-MLRQ). [Source:Uniprot/SWISSPROT;Acc:O00483]	4846	593	-3.03
ART1	GPI-linked NADP(+)--arginine ADP-ribosyltransferase 1 precursor (EC 2.4.2.31) (Mono(ADP-ribosyl)transferase) (CD296 antigen). [Source:Uniprot/SWISSPROT;Acc:P52961]	8	1	-3
NP_001001684.1	CDNA FLJ45831 fis, clone NT2RP8007416. [Source:Uniprot/SPTREMBL;Acc:Q6ZS49]	8	1	-3
AKAP3	A-kinase anchor protein 3 (Protein kinase A-anchoring protein 3) (PRKA3) (A-kinase anchor protein 110 kDa) (AKAP 110) (Sperm oocyte- binding protein) (Fibrousheathin-1) (Fibrousheathin I) (Fibrous sheath protein of 95 kDa) (FSP95). [Source:Uniprot/SWISSPROT;Acc:O75969]	8	1	-3
GTF2IRD2	GTF2I repeat domain containing 2 [Source:RefSeq_peptide;Acc:NP_775808]	8	1	-3
SPATA16	spermatogenesis associated 16 [Source:RefSeq_peptide;Acc:NP_114161]	23	3	-2.94
SPINLW1	Eppin precursor (Epididymal protease inhibitor) (Serine protease inhibitor-like with Kunitz and WAP domains 1) (WAP four-disulfide core domain protein 7) (Protease inhibitor WAP7). [Source:Uniprot/SWISSPROT;Acc:O95925]	23	3	-2.94
OR2L2	Olfactory receptor 2L2 (HTPCRH07). [Source:Uniprot/SWISSPROT;Acc:Q8NH16]	23	3	-2.94
HTR5A	5-hydroxytryptamine 5A receptor (5-HT-5A) (Serotonin receptor 5A) (5- HT-5). [Source:Uniprot/SWISSPROT;Acc:P47898]	30	4	-2.91
CF015_HUMAN	[Source:Uniprot/SWISSPROT;Acc:Q6UXA7]	30	4	-2.91
Q9H7Y2_HUMAN	CDNA FLJ14100 fis, clone MAMMA1000855. [Source:Uniprot/SPTREMBL;Acc:Q9H7Y2]	30	4	-2.91
ZFP42	zinc finger protein 42 [Source:RefSeq_peptide;Acc:NP_777560]	15	2	-2.91
C1orf112	C1orf112 protein. [Source:Uniprot/SPTREMBL;Acc:Q3KNQ1]	15	2	-2.91
EDAR	Tumor necrosis factor receptor superfamily member EDAR precursor (Anhidrotic ectodysplasin receptor 1) (Ectodysplasin-A receptor) (EDA- A1 receptor) (Ectodermal dysplasia receptor) (Downless homolog). [Source:Uniprot/SWISSPROT;Acc:Q9UNE0]	15	2	-2.91
RNF11	RING finger protein 11 (Sid 1669). [Source:Uniprot/SWISSPROT;Acc:Q9Y3C5]	949	129	-2.88
CLDN14	Claudin-14. [Source:Uniprot/SWISSPROT;Acc:O95500]	22	3	-2.87
SGCZ	Zeta-sarcoglycan (Zeta-SG) (ZSG1). [Source:Uniprot/SWISSPROT;Acc:Q96LD1]	29	4	-2.86
PLAC8	Placenta-specific gene 8 protein (Protein C15). [Source:Uniprot/SWISSPROT;Acc:Q9NZF1]	121	17	-2.83
LRRC17	[Source:Uniprot/SWISSPROT;Acc:Q8N6Y2]	7	1	-2.81
PCDH7	[Source:Uniprot/SWISSPROT;Acc:O60245]	35	5	-2.81
TRAV22	T-cell receptor alpha V gene segment [Source:IMGT/GENE-DB;Acc:TRAV22]	7	1	-2.81
XAB2	XPA-binding protein 2 (HCNP protein). [Source:Uniprot/SWISSPROT;Acc:Q9HCS7]	35	5	-2.81
FCGR2A	(IgG Fc receptor II-a) (Fc-gamma-RIIa) (CD32 antigen) (CDw32). [Source:Uniprot/SWISSPROT;Acc:P12318]	21	3	-2.81
SEC14L5	-	21	3	-2.81
C10orf53	Uncharacterized protein C10orf53. [Source:Uniprot/SWISSPROT;Acc:Q8N6V4]	21	3	-2.81
LOC729516	-	21	3	-2.81
SLFNL1	schlafen-like 1 [Source:RefSeq_peptide;Acc:NP_659427]	14	2	-2.81

Supplementary Table 2. The list represents 222 genes decreased by more than five-fold in miR-210-tansfected cells compared with ncRNA-tansfected cells				
Gene Symbol	**description**	**ncRNA**	**miR-210**	**Log_2(ratio)**
LRAT	Lecithin retinol acyltransferase (EC 2.3.1.135) (Phosphatidylcholine-- retinol O-acyltransferase). [Source:Uniprot/SWISSPROT;Acc:O95237]	7	1	-2.81
GPR177	Integral membrane protein GPR177 precursor (Protein wntless homolog) (Putative NFkB-activating protein 373). [Source:Uniprot/SWISSPROT;Acc:Q5T9L3]	817	121	-2.76
ADAMTS13	ADAMTS-13 precursor (EC 3.4.24.-) (A disintegrin and metalloproteinase with thrombospondin motifs 13) (ADAM-TS 13) (ADAM-TS13) (von Willebrand factor-cleaving protease) (vWF-cleaving protease) (vWF-CP). [Source:Uniprot/SWISSPROT;Acc:Q76LX8]	47	7	-2.75
PTCHD1	patched domain containing 1 [Source:RefSeq_peptide;Acc:NP_775766]	20	3	-2.74
ACPL2	acid phosphatase-like 2 [Source:RefSeq_peptide;Acc:NP_0010322-49]	20	3	-2.74
TFEC	transcription factor EC isoform b [Source:RefSeq_peptide;Acc:NP_0010180-68]	33	5	-2.72
STMN1	phosphoprotein p18) (pp17) (Prosolin) (Metablastin) (Protein Pr22). [Source:Uniprot/SWISSPROT;Acc:P16949]	1003	153	-2.71
Q8WYR5_HUMAN	Tumor rejection antigen. [Source:Uniprot/SPTREMBL;Acc:Q8WYR5]	557	85	-2.71
Q8NHT0_HUMAN	MGC32805 protein. [Source:Uniprot/SPTREMBL;Acc:Q8NHT0]	26	4	-2.7
C20orf39	UPF0338 protein C20orf39. [Source:Uniprot/SWISSPROT;Acc:Q9H7V2]	26	4	-2.7
PSG10	Pregnancy-specific beta-1-glycoprotein 10 precursor (PSBG-10) (PSBG- 12). [Source:Uniprot/SWISSPROT;Acc:Q15235]	13	2	-2.7
GJA5	Gap junction alpha-5 protein (Connexin-40) (Cx40). [Source:Uniprot/SWISSPROT;Acc:P36382]	13	2	-2.7
POT15_HUMAN	[Source:Uniprot/SWISSPROT;Acc:Q6S5H4]	13	2	-2.7
NP_001008784.1	CD200 cell surface glycoprotein receptor isoform 2 [Source:RefSeq_peptide;Acc:NP_001008784]	13	2	-2.7
PER3	Period circadian protein homolog 3 (Circadian clock protein PERIOD 3) (hPER3). [Source:Uniprot/SWISSPROT;Acc:P56645]	45	7	-2.68
TEDDM1	putative membrane protein HE9 [Source:RefSeq_peptide;Acc:NP_741997]	44	7	-2.65
DOCK4	Dedicator of cytokinesis protein 4. [Source:Uniprot/SWISSPROT;Acc:Q8N110]	25	4	-2.64
RIMS3	Regulating synaptic membrane exocytosis protein 3 (Nim3) (Rab-3- interacting molecule 3) (RIM 3) (RIM3 gamma). [Source:Uniprot/SWISSPROT;Acc:Q9UJD0]	43	7	-2.62
FCRL3	Fc receptor-like 3 precursor [Source:RefSeq_peptide;Acc:NP_443171]	49	8	-2.61
GCKR	Glucokinase regulatory protein (Glucokinase regulator). [Source:Uniprot/SWISSPROT;Acc:Q14397]	6	1	-2.58
NP_631912.2	Na+/H+ exchanger like domain containing [Source:RefSeq_peptide;Acc:NP_631912]	24	4	-2.58
C2orf51	CDNA FLJ25369 fis, clone TST01830 (Hypothetical protein FLJ25369) (Chromosome 2 open reading frame 51). [Source:Uniprot/SPTREMBL;Acc:Q96LM6]	48	8	-2.58
EN2	Homeobox protein engrailed-2 (Hu-En-2). [Source:Uniprot/SWISSPROT;Acc:P19622]	30	5	-2.58
RFPL1	Ret finger protein-like 1 (RING finger protein 78). [Source:Uniprot/SWISSPROT;Acc:O75677]	30	5	-2.58
GDF3	Growth/differentiation factor 3 precursor (GDF-3). [Source:Uniprot/SWISSPROT;Acc:Q9NR23]	36	6	-2.58
NP_775916.1	CDNA FLJ37357 fis, clone BRAMY2023060 (FLJ37357 protein) (Hypothetical protein FLJ37357). [Source:Uniprot/SPTREMBL;Acc:Q8N1W6]	30	5	-2.58
ISX	intestine-specific homeobox [Source:RefSeq_peptide;Acc:NP_001008494]	24	4	-2.58
CX3CR1	CX3C chemokine receptor 1 (C-X3-C CKR-1) (CX3CR1) (Fractalkine receptor) (G-protein coupled receptor 13) (V28) (Beta chemokine receptor-like 1) (CMK-BRL-1) (CMKBLR1). [Source:Uniprot/SWISSPROT;Acc:P49238]	24	4	-2.58
MIA2	Melanoma inhibitory activity protein 2 precursor. [Source:Uniprot/SWISSPROT;Acc:Q96PC5]	18	3	-2.58
O10D4_HUMAN	Olfactory receptor 10D4. [Source:Uniprot/SWISSPROT;Acc:Q8NGN7]	18	3	-2.58
OR7G2	Olfactory receptor 7G2 (Olfactory receptor 19-13) (OR19-13) (OST260). [Source:Uniprot/SWISSPROT;Acc:Q8NG99]	18	3	-2.58
LRP2	Low-density lipoprotein receptor-related protein 2 precursor (Megalin) (Glycoprotein 330) (gp330). [Source:Uniprot/SWISSPROT;Acc:P98164]	12	2	-2.58
C2orf46	Putative uncharacterized protein C2orf46. [Source:Uniprot/SWISSPROT;Acc:Q6ZSB3]	12	2	-2.58
Q6ZMT9_HUMAN	[Source:Uniprot/SPTREMBL;Acc:Q6ZMT9]	6	1	-2.58
C1QTNF2	Complement C1q tumor necrosis factor-related protein 2 precursor. [Source:Uniprot/SWISSPROT;Acc:Q9BXJ5]	6	1	-2.58
SERHL2	Serine hydrolase-like protein 2 (EC 3.1.-.-). [Source:Uniprot/SWISSPROT;Acc:Q9H4I8]	64	11	-2.54
USHBP1	USH1C-binding protein 1 (Usher syndrome type-1C protein-binding protein 1) (MCC-2) (AIE-75-binding protein). [Source:Uniprot/SWISSPROT;Acc:Q8N6Y0]	29	5	-2.54
VSTM2	[Source:Uniprot/SWISSPROT;Acc:Q8TAG5]	23	4	-2.52
AGC1	sulfate proteoglycan core protein 1) [Contains: Aggrecan core protein 2]. [Source:Uniprot/SWISSPROT;Acc:P16112]	23	4	-2.52
BPY2C	[Source:Uniprot/SWISSPROT;Acc:O14599]	23	4	-2.52
DDOST	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit precursor (EC 2.4.1.119) (Oligosaccharyl transferase 48 kDa subunit) (DDOST 48 kDa subunit). [Source:Uniprot/SWISSPROT;Acc:P39656]	2238	391	-2.52
IRX1	Iroquois-class homeodomain protein IRX-1 (Iroquois homeobox protein 1) (Homeodomain protein IRXA1). [Source:Uniprot/SWISSPROT;Acc:P78414]	34	6	-2.5
CD4	T-cell surface glycoprotein CD4 precursor (T-cell surface antigen T4/Leu-3). [Source:Uniprot/SWISSPROT;Acc:P01730]	17	3	-2.5
Q8NAH5_HUMAN	CDNA FLJ35343 fis, clone PROST2015932. [Source:Uniprot/SPTREMBL;Acc:Q8NAH5]	17	3	-2.5
PSG2	Pregnancy-specific beta-1-glycoprotein 2 precursor (PSBG-2) (Pregnancy-specific beta-1 glycoprotein E) (PS-beta-E). [Source:Uniprot/SWISSPROT;Acc:P11465]	28	5	-2.49
THBS1	Thrombospondin-1 precursor. [Source:Uniprot/SWISSPROT;Acc:P07996]	206	37	-2.48
ANKRD20A3	Ankyrin repeat domain-containing protein 20A3. [Source:Uniprot/SWISSPROT;Acc:Q5VUR7]	22	4	-2.46

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Gene Symbol	description	ncRNA	miR-210	Log$_2$(ratio)
FNBP1L	Formin-binding protein 1-like (Transducer of Cdc42-dependent actin assembly protein 1) (Toca-1). [Source:Uniprot/SWISSPROT;Acc:Q5T0N5]	33	6	-2.46
HBB	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin). [Source:Uniprot/SWISSPROT;Acc:P68871]	22	4	-2.46
PIWIL3	Piwi-like protein 3. [Source:Uniprot/SWISSPROT;Acc:Q7Z3Z3]	22	4	-2.46
MAGEA11	Melanoma-associated antigen 11 (MAGE-11 antigen). [Source:Uniprot/SWISSPROT;Acc:P43364]	22	4	-2.46
KCNG1	Potassium voltage-gated channel subfamily G member 1 (Voltage-gated potassium channel subunit Kv6.1) (kH2). [Source:Uniprot/SWISSPROT;Acc:Q9UIX4]	11	2	-2.46
ADRA1A	Alpha-1A adrenergic receptor (Alpha 1A-adrenoceptor) (Alpha 1A- adrenoreceptor) (Alpha-1C adrenergic receptor) (Alpha adrenergic receptor 1c). [Source:Uniprot/SWISSPROT;Acc:P35348]	11	2	-2.46
NP_001001343.1	MGC27121 gene (MGC27121), mRNA [Source:RefSeq_dna;Acc:NM_001001343]	38	7	-2.44
GPNMB	Transmembrane glycoprotein NMB precursor (Transmembrane glycoprotein HGFIN). [Source:Uniprot/SWISSPROT;Acc:Q14956]	54	10	-2.43
C1orf173	C1orf173 protein. [Source:Uniprot/SPTREMBL;Acc:Q6GMR8]	27	5	-2.43
NTS	Neurotensin/neuromedin N precursor [Contains: Large neuromedin N (NmN- 125); Neuromedin N (NmN) (NN); Neurotensin (NT); Tail peptide]. [Source:Uniprot/SWISSPROT;Acc:P30990]	27	5	-2.43
XKR4	XK-related protein 4. [Source:Uniprot/SWISSPROT;Acc:Q5GH76]	16	3	-2.42
C12orf59	C12orf59 protein. [Source:Uniprot/SPTREMBL;Acc:Q4KMG9]	159	30	-2.41
USP29	specific-processing protease 29) (Deubiquitinating enzyme 29). [Source:Uniprot/SWISSPROT;Acc:Q9HBJ7]	21	4	-2.39
PLA2G7	acylhydrolase) (LDL-associated phospholipase A2) (LDL-PLA(2)) (2-acetyl-1-alkylglycerophosphocholine esterase) (1-alkyl-2-acetylglycerophosphocholine esterase) [Source:Uniprot/SWISSPROT;Acc:Q13093]	21	4	-2.39
NP_001006656.1	MSTP119. [Source:Uniprot/SPTREMBL;Acc:Q7Z2S5]	21	4	-2.39
CEECAM1	cerebral endothelial cell adhesion molecule 1 [Source:RefSeq_peptide;Acc:NP_057258]	21	4	-2.39
ZDHHC22	Putative palmitoyltransferase ZDHHC22 (EC 2.3.1.-) (Zinc finger DHHC domain-containing protein 22) (DHHC-22). [Source:Uniprot/SWISSPROT;Acc:Q8N966]	21	4	-2.39
ZNF101	Zinc finger protein 101 (Zinc finger protein HZF12). [Source:Uniprot/SWISSPROT;Acc:Q8IZC7]	47	9	-2.38
ADRA1B	Alpha-1B adrenergic receptor (Alpha 1B-adrenoceptor) (Alpha 1B- adrenoreceptor). [Source:Uniprot/SWISSPROT;Acc:P35368]	26	5	-2.38
ACOX3	Acyl-coenzyme A oxidase 3, peroxisomal (EC 1.3.3.6) (Pristanoyl-CoA oxidase) (Branched-chain acyl-CoA oxidase) (BRCACox). [Source:Uniprot/SWISSPROT;Acc:O15254]	31	6	-2.37
Q8N7S0_HUMAN	CDNA FLJ40424 fis, clone TESTI2039026. [Source:Uniprot/SPTREMBL;Acc:Q8N7S0]	31	6	-2.37
PHKG1	Phosphorylase b kinase gamma catalytic chain, skeletal muscle isoform (EC 2.7.11.19) (Phosphorylase kinase subunit gamma 1). [Source:Uniprot/SWISSPROT;Acc:Q16816]	31	6	-2.37
LRP5L	low density lipoprotein receptor-related protein 5-like [Source:RefSeq_peptide;Acc:NP_872298]	67	13	-2.37
AAK1	AP2-associated protein kinase 1 (EC 2.7.11.1) (Adaptor-associated kinase 1). [Source:Uniprot/SWISSPROT;Acc:Q2M2I8]	221	43	-2.36
KIAA0195	KIAA0195 (KIAA0195), mRNA [Source:RefSeq_dna;Acc:NM_014738]	40	8	-2.32
Q8NA59_HUMAN	CDNA FLJ35816 fis, clone TESTI2006109. [Source:Uniprot/SPTREMBL;Acc:Q8NA59]	30	6	-2.32
EMID1	EMI domain-containing protein 1 precursor (Protein Emu1) (Emilin and multimerin domain-containing protein 1). [Source:Uniprot/SWISSPROT;Acc:Q96A84]	5	1	-2.32
C6orf122	Uncharacterized protein C6orf122. [Source:Uniprot/SWISSPROT;Acc:Q5T6M2]	20	4	-2.32
ERC1	ELKS/RAB6-interacting/CAST family member 1 (RAB6-interacting protein 2) (ERC protein 1). [Source:Uniprot/SWISSPROT;Acc:Q8IUD2]	20	4	-2.32
USP6	Ubiquitin carboxyl-terminal hydrolase 6 (EC 3.1.2.15) (Ubiquitin thioesterase 6) (Ubiquitin-specific-processing protease 6) (Deubiquitinating enzyme 6) (Proto-oncogene TRE-2). [Source:Uniprot/SWISSPROT;Acc:P35125]	20	4	-2.32
GRB14	Growth factor receptor-bound protein 14 (GRB14 adapter protein). [Source:Uniprot/SWISSPROT;Acc:Q14449]	15	3	-2.32
ALG10	(Asparagine-linked glycosylation protein 10 homolog A). [Source:Uniprot/SWISSPROT;Acc:Q5BKT4]	15	3	-2.32
SPTB	Spectrin beta chain, erythrocyte (Beta-I spectrin). [Source:Uniprot/SWISSPROT;Acc:P11277]	15	3	-2.32
C3AR1	[Source:Uniprot/SWISSPROT;Acc:Q16581]	10	2	-2.32
HTR2C	5-hydroxytryptamine 2C receptor (5-HT-2C) (Serotonin receptor 2C) (5- HT2C) (5-HTR2C) (5HT-1C). [Source:Uniprot/SWISSPROT;Acc:P28335]	10	2	-2.32
Q95724_HUMAN	Reverse transcriptase (Fragment). [Source:Uniprot/SPTREMBL;Acc:O95724]	5	1	-2.32

The fifteen genes predicted as a miR-210 target gene by microCosm, TargetScan or PicTar were highlighted in gray.

Supplementary Table 3. The list represents 811 genes predicted as a miR-210 target gene by microCosm, TargetScan and PicTar

ABCC1	*C10orf122*	*CD8A*	*EFNA3*	*GIT2*	*IMP3*
ABCC6P2	*C11orf2*	*CD99*	*EGFL9*	*GJA7*	*IQCK*
ABCD1	*C11orf58*	*CDC2L1*	*EGR3*	*GLI1*	*IRGC*
ABCD4	*C11orf59*	*CDC2L2*	*EGR4*	*GLIS1*	*IRX6*
ABHD2	*C11orf63*	*CDH26*	*EHD2*	*GLS2*	*ISCA2*
ABL2	*C12orf34*	*CDKN1C*	*EIF3S9*	*GNA15*	*ISCU*
ABTB1	*C12orf48*	*CDX2*	*ELA2*	*GNAT1*	*ITGA8*
AC006273.1	*C14orf148*	*CEND1*	*ELAC2*	*GNG3*	*ITGB4*
AC008772.1	*C15orf43*	*CETN3*	*ELFN2*	*GNG8*	*IWS1*
AC008898.1	*C15orf52*	*CFHR2*	*ELOVL6*	*GOLGA1*	*JOSD2*
AC009967.5	*C15orf62*	*CHAD*	*EMID1*	*GOLGA2LY1*	*KAAG1*
AC020916.6	*C16orf35*	*CHD1L*	*EML2*	*GOLPH3*	*KCMF1*
AC040977.2	*C16orf70*	*CHEK2*	*ENPP2*	*GPD1L*	*KCNH5*
AC109322.1	*C17orf57*	*CHES1*	*ENSA*	*GPR153*	*KCNMB1*
AC114498.5	*C17orf64*	*CHN1*	*EPB41L1*	*GPR17*	*KCNN2*
AC133485.1	*C17orf83*	*CHRDL1*	*EPGN*	*GPR177*	*KCNQ2*
AC141586.2	*C18orf10*	*CHRM3*	*EPHA2*	*GPR19*	*KIAA0664*
AC226119.1	*C18orf34*	*CHRNB1*	*EPHB2*	*GPR39*	*KIAA0748*
ACP1	*C19orf16*	*CHRNG*	*EPS15*	*GPR87*	*KIAA1622*
ACTA1	*C19orf34*	*CHST1*	*ERP27*	*GRIA2*	*KIAA1751*
ACTC1	*C19orf59*	*CHST12*	*EVL*	*GRIK2*	*KIAA1755*
ACTG1	*C1orf102*	*CHUK*	*EVPL*	*GRIN3B*	*KIAA2013*
ACTL7A	*C1orf110*	*CIRBP*	*EXOSC10*	*GRINL1A*	*KIF13B*
ADA	*C1orf111*	*CLDN15*	*F11R*	*GRM5*	*KIF20A*
ADAMTS7	*C1orf112*	*CLEC11A*	*F7*	*GRM6*	*KIR2DL1*
ADCY7	*C1orf116*	*CLEC19A*	*FAHD1*	*GSC*	*KLHDC4*
AGPAT2	*C1orf128*	*CNGB1*	*FAM105B*	*GSTA1*	*KLHL35*
AGTRAP	*C1orf133*	*COL16A1*	*FAM108A1*	*GSTA2*	*KLRA1*
AGTRL1	*C1orf167*	*COL4A2*	*FAM108A2*	*GSTTP2*	*KLRC4*
AGXT2L2	*C1orf184*	*COL4A3*	*FAM116A*	*GSTZ1*	*KRTAP5-4*
AIHR	*C1orf86*	*COL6A2*	*FAM120A*	*H2AFY*	*LAIR2*
AIFM3	*C1orf88*	*COL9A3*	*FAM12A*	*HAAO*	*LAMA3*
AKAP7	*C1QTNF4*	*COMMD4*	*FAM149A*	*HAS1*	*LAMA5*
AKAP9	*C1S*	*COX10*	*FAM25A*	*HCN4*	*LAMC3*
AKR1CL2	*C20orf103*	*CPNE2*	*FAM25B*	*HDLBP*	*LCE3E*
AL132661.1	*C20orf195*	*CR1*	*FAM25C*	*HEATR7B2*	*LCK*
AL161662.2	*C2orf32*	*CREB3L3*	*FAM5B*	*HELZ*	*LCN8*
ALDH3A1	*C2orf46*	*CRHBP*	*FAM73B*	*HHIP*	*LDHB*
ALDH4A1	*C3orf21*	*CRLF3*	*FAM84A*	*HIF3A*	*LEAP2*
ALKBH3	*C3orf30*	*CRYBA4*	*FAM90A1*	*HIRIP3*	*LGI3*
ALLC	*C4orf23*	*CS*	*FAM90A10*	*HIST1H1B*	*LHPP*
AMBN	*C6orf125*	*CSPP1*	*FAM90A18*	*HIST1H2AK*	*LIPE*
ANAPC7	*C6orf145*	*CST3*	*FAM90A3*	*HK3*	*LIPI*
ANK1	*C6orf184*	*CTBP2*	*FAM90A7*	*HLXB9*	*LMAN1L*
ANKRD24	*C6orf191*	*CUEDC2*	*FAM90A8*	*HMG20B*	*LMTK2*
ANKRD47	*C6orf57*	*CUL9*	*FAM90A9*	*HOXA3*	*LMX1B*
ANKS3	*C9*	*CYB5R2*	*FAM9B*	*HPCA*	*LOC399947*
AP000355.2	*C9orf129*	*CYGB*	*FANCB*	*HPCAL1*	*LOC643224*
AP000889.1	*C9orf134*	*CYP11B1*	*FANCE*	*HRH2*	*LRFN1*
APOBEC3G	*C9orf78*	*CYP2F1*	*FANK1*	*HSD17B1*	*LRP5L*
ARFRP1	*C9orf85*	*DAB1*	*FBXL12*	*HSD17B7*	*LRPAP1*
ARHGAP17	*C9orf86*	*DARC*	*FBXL16*	*HSPA8*	*LRRC62*
ARHGEF17	*C9orf93*	*DBN1*	*FBXL17*	*HSPBAP1*	*LRRC68*
ARMC1	*CACNA2D2*	*DCHS1*	*FBXW5*	*HTATSF1*	*LRRC8A*
ARMC4	*CALCOCO1*	*DCTN1*	*FER1L4*	*HTRA1*	*LRRC8D*
ARSD	*CAMK2G*	*DDX24*	*FEZF1*	*HYAL1*	*LRRC8D*
ASCC1	*CAPN10*	*DDX51*	*FGD1*	*ID12*	*LRSAM1*
ASCL1	*CAPN9*	*DEAF1*	*FGF10*	*IGF1R*	*LY6H*
ASL	*CARD9*	*DEC1*	*FGF18*	*IGHA2*	*LYN*
ATG4D	*CASQ1*	*DEFB118*	*FGF22*	*IGHV3-13*	*LYPLA2*
ATN1	*CCBP2*	*DGKA*	*FGFRL1*	*IGHV3-16*	*MAGI2*
ATP12A	*CCDC146*	*DGKG*	*FHIT*	*IGHV3-23*	*MAN1B1*
ATP1B1	*CCDC17*	*DHRS3*	*FHOD1*	*IGHV3-35*	*MAP6*
ATP2B3	*CCDC24*	*DHX58*	*FKBP9L*	*IGHV3-38*	*MAP7D1*
ATP6V0C	*CCDC28A*	*DIMT1L*	*FMO1*	*IGHV3-47*	*MARCH4*
AVP	*CCDC28B*	*DNAH11*	*FNTA*	*IGHV3-66*	*MARK1*
AVPR1B	*CCDC53*	*DNAJC16*	*FOXD2*	*IGKV4-1*	*MAST1*
B3GALT5	*CCDC95*	*DNAJC4*	*FOXP3*	*IGLC7*	*MBD5*
B4GALT5	*CCDC97*	*DNAJC8*	*FRAP1*	*IGLL3*	*MCCD1*
B4GALT7	*CCKBR*	*DPEP2*	*FRY*	*IGSF21*	*MCM4*
BAIAP3	*CCNB1IP1*	*DPYSL5*	*FTSJ2*	*IHPK2*	*MCM8*
BCAS2	*CCNL2*	*DRD5*	*FUT8*	*IHPK3*	*MDGA1*
BCL2L12	*CCT3*	*DTX1*	*G0S2*	*IKBKG*	*MEF2D*
BDNF	*CD180*	*DUOXA1*	*GAK*	*IL1RA*	*MEGF6*
BICD1	*CD22*	*DUSP12*	*GARNL1*	*IL17RC*	*MEIS1*
BRP44L	*CD276*	*DUSP28*	*GAS6*	*IL18*	*MFAP3*
BTBD10	*CD300LD*	*E2F3*	*GBGT1*	*IL3RA*	*MFSD4*
BX324178.1	*CD55*	*EBF3*	*GDAP1L1*	*ILVBL*	*MID1IP1*

Supplementary Table 3. The list represents 811 genes predicted as a miR-210 target gene by microCosm, TargetScan and PicTar

MINA	*PCP4*	*RP4-565E6.1*	*SURF5*	*WNT9B*
MLL2	*PCSK7*	*RPS27*	*SUSD1*	*XCR1*
MPV17L	*PDCL*	*RSC1A1*	*SUZ12*	*XKR5*
MRPL36	*PDE3A*	*RUFY1*	*SYNE2*	*XRRA1*
MRPS30	*PDLIM3*	*RUVBL2*	*SYNGAP1*	*YIPF3*
MS4A8B	*PDX1*	*SACM1L*	*SYT15*	*YY1*
MT4	*PDZD4*	*SAMD13*	*TAC4*	*Z97180.2*
MUC4	*PEX10*	*SAP30L*	*TAF6*	*ZBTB12*
MX1	*PEX13*	*SARNP*	*TBC1D16*	*ZCCHC11*
MXD4	*PEX5L*	*SCGB1C1*	*TBC1D28*	*ZDHHC12*
MYH11	*PHF15*	*SCN1B*	*TBC1D8*	*ZDHHC4*
MYO15B	*PHF23*	*SCN9A*	*TCF7L2*	*ZFPM1*
MYOHD1	*PHKG2*	*SCOC*	*TCHP*	*ZHX2*
MYT1L	*PHTF1*	*SCRT1*	*TFAP2A*	*ZMAT4*
NAT14	*PI1KAP2*	*SCYL1*	*TFCP2*	*ZMIZ2*
NDUFA4	*PIK3R5*	*SDCCAG8*	*TGFBRAP1*	*ZMYM2*
NEFM	*PIK4CA*	*SDF2*	*THOC5*	*ZMYM6*
NEK3	*PKNOX2*	*SDHALP1*	*TIAM1*	*ZNF227*
NEUROD2	*PLA1A*	*SDHALP2*	*TIGD2*	*ZNF274*
NEUROG3	*PLCB3*	*SEC24C*	*TIGD5*	*ZNF397*
NF1B	*PLCD3*	*SEH1L*	*TIMM17B*	*ZNF403*
NFIL3	*PLK1*	*SENP8*	*TIMP1*	*ZNF418*
NKX2-5	*PLXNC1*	*SEPT12*	*TLX1*	*ZNF45*
NKX2-8	*PODN*	*SERPINA12*	*TMEM142A*	*ZNF462*
NOMO1	*POF1B*	*SERPINA3*	*TMEM16B*	*ZNF467*
NOMO3	*POLR2I*	*SETD2*	*TMEM16D*	*ZNF583*
NOX4	*POU2AF1*	*SF3B1*	*TMEM194B*	*ZNF585B*
NP_660343.1	*PPAP2A*	*SF3B4*	*TMEM195*	*ZNF720*
NP_683701.2	*PPIE*	*SF3B5*	*TMEM204*	*ZNF827*
NPHP4	*PPP1CC*	*SH2D7*	*TMEM208*	*ZRANB3*
NPM3	*PPP1R12C*	*SH3BGR*	*TMEM40*	*ZSCAN20*
NPR1	*PPP1R16A*	*SH3BGRL*	*TNFRSF13C*	
NPSR1	*PPP3R1*	*SH3KBP1*	*TNIP1*	
NPTX1	*PRHOXNB*	*SHC3*	*TNIP3*	
NQO2	*PRICKLE4*	*SHCBP1*	*TNPO3*	
NR6A1	*PRIMA1*	*SIDT2*	*TOM1L2*	
NRG4	*PRMT2*	*SIL1*	*TOR1A*	
NRGN	*PRPF38B*	*SIN3B*	*TP53TG3*	
NSDHL	*PRR11*	*SIPA1L3*	*TPST1*	
NSUN5	*PRRG2*	*SIX1*	*TRAF3D1*	
NT5C1B	*PRSS16*	*SLC12A8*	*TRAV4*	
NT5DC1	*PSG2*	*SLC15A1*	*TRIM14*	
NTN4	*PSME3*	*SLC16A14*	*TRIM17*	
NUDT6	*PTAFR*	*SLC20A1*	*TSEN2*	
NUP133	*PTCHD3*	*SLC25A28*	*TSNAXIP1*	
NUPL2	*PTGES2*	*SLC26A3*	*TSPAN10*	
NUPR1	*PTS*	*SLC2A1*	*TSPAN14*	
NYX	*PYDC1*	*SLC37A1*	*TTC12*	
OAF	*PYY*	*SLC38A5*	*TTC13*	
OBSCN	*Q6ZRP6_HUMAN*	*SLC43A1*	*TTC24*	
ODF3	*Q9BZU2_HUMAN*	*SLC4A11*	*TTC4*	
ODZ2	*Q9HBR8_HUMAN*	*SLC6A19*	*TTF1*	
ODZ3	*RAB26*	*SLITRK5*	*TTLL1*	
OGG1	*RAB27B*	*SMARCA4*	*TXNL6*	
OLFML2A	*RABGAP1L*	*SMCHD1*	*UBASH3A*	
OLIG3	*RAD52*	*SORBS2*	*UBE2O*	
OR10G8	*RALGDS*	*SOX21*	*UBN2*	
OR2T8	*RANBP5*	*SOX30*	*UBQLN1*	
OR2V2	*RASAL2*	*SPACA3*	*UMODL1*	
OR4K14	*RASSF1*	*SPACA4*	*UNC45A*	
OR4P1P	*RASSF6*	*SPO11*	*UNC5A*	
OR5M13P	*RBM3*	*SPRR2F*	*URM1*	
OSBPL2	*RBMY1C*	*SPTB*	*UROS*	
OTOS	*RBPJL*	*SQLE*	*USP21*	
OTP	*RC3H1*	*SREBF1*	*USP6NL*	
OTUB1	*RECK*	*SRMS*	*VIT*	
P2RX1	*RETN*	*SRPX*	*VRK1*	
P2RY10	*RG9MTD1*	*ST3GAL3*	*VSIG6*	
P2RY11	*RGS4*	*ST6GALNAC6*	*VWA5B1*	
P4HA3	*RIBC2*	*STAB1*	*WDFY2*	
PABPC1L	*RLN3*	*STARD3NL*	*WDR20*	
PADI1	*RNF207*	*STAT6*	*WDR22*	
PANX3	*RNF208*	*STIP1*	*WDR38*	
PAOX	*RNF212*	*STT3B*	*WDR5B*	
PARK2	*RP1-163G9.1*	*STX11*	*WDR6*	
PBX1	*RP11-181G12.1*	*STX1B2*	*WDR64*	
PCBP4	*RP11-191L9.1*	*STXBP5*	*WDR66*	
PCDH21	*RP11-397P14.1*	*SUMO3*	*WISP2*	

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ORIGINAL ARTICLE

MicroRNA-141 confers resistance to cisplatin-induced apoptosis by targeting *YAP1* in human esophageal squamous cell carcinoma

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MicroRNAs (miRNAs) are endogenous non-coding RNAs that function as negative regulators of gene expression. Alterations in miRNA expression have been shown to affect tumor growth and response to chemotherapy. In this study, we explored the possible role of miRNAs in cisplatin resistance in esophageal squamous cell carcinoma (ESCC). First we assessed the sensitivity of nine human ESCC cell lines (KYSE series) to cisplatin using an *in vitro* cell viability assay, and then we compared the miRNA profiles of the cisplatin-sensitive and -resistant cell lines by miRNA microarray analysis. The two groups showed markedly different miRNA expression profiles, and 10 miRNAs were found to be regulated differentially between the two groups. When miR-141, which was the most highly expressed miRNA in the cisplatin-resistant cell lines, was expressed ectopically in the cisplatin-sensitive cell lines, cell viability after cisplatin treatment was increased significantly. Furthermore, we found that miR-141 directly targeted the 3′-untranslated region of *YAP1*, which is known to have a crucial role in apoptosis induced by DNA-damaging agents, and thus downregulated *YAP1* expression. Our study highlights an important regulatory role for miR-141 in the development of cisplatin resistance in ESCC.
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Keywords: apoptosis; cisplatin resistance; esophageal carcinoma; microRNA; *YAP1*

INTRODUCTION

MicroRNAs (miRNAs) are a class of small (~22 bp) endogenous non-coding RNAs that are well conserved, and function as negative regulators of gene expression. miRNAs bind to complementary sequences in the 3′-untranslated region (UTR) of target messenger RNAs and regulate their expression by cleavage and/or translational inhibition.[1] miRNAs are predicted to regulate the expression of up to one-third of human protein-coding genes,[2–5] and they have been shown to have crucial roles in diverse biological processes, including development, differentiation, apoptosis and proliferation.[6–8] A growing number of studies have provided strong evidence that aberrant miRNA expression is involved in the genesis and progression of cancer,[9] and that miRNAs might function as a novel class of oncogenes or tumor-suppressor genes.[10–13] Altered expression of miRNAs in primary human cancers has been used for tumor diagnosis, classification, staging and prognosis.[14] Furthermore, the involvement of miRNAs in the response of tumor cells to chemotherapeutic agents has also been confirmed,[15–17] which suggests that miRNAs could have a broad effect on the response of cancer cells to chemotherapy.

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer deaths worldwide.[18] In spite of comprehensive available treatment, including chemotherapy, surgery and radiotherapy, the overall 5-year survival rate for patients with esophageal squamous cell carcinoma (ESCC), the most common form of esophageal cancer, remains low, at 10–40%, because of advanced disease, metastasis and resistance of the tumor to chemotherapy and radiotherapy.[19–21] Cisplatin is the most frequently used chemotherapeutic agent for ESCC. However, given that resistance to cisplatin limits the success of treatment, elucidation of the mechanisms that regulate cisplatin resistance in ESCC is urgently needed. In the present study, we studied the biological function of miRNAs in the development of cisplatin resistance in ESCC using the KYSE series of ESCC cell lines as a model, and focused, in particular, on the regulation of apoptosis.

MATERIALS AND METHODS

Cell lines and cultures

Human KYSE cell lines that had been established from primary tumors at our institution as described previously were cultured in RPMI 1640

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