# High expression of microRNA-127 is involved in cell cycle arrest in MC-3 mucoepidermoid carcinoma cells

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**Abstract.** microRNAs (miRs) are small endogenous non-coding RNAs and are associated with the pathogenesis of a number of types of human cancer. However, miR-127-3p in mucoepidermoid carcinoma (MEC) has not been studied. The present study aimed to analyze the importance of miR-127-3p in MC-3 human MEC cells. Analyses of the growth inhibitory effect and the associated mechanism of miR-127-3p were performed using 3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium assay, flow cytometry, 4'-6-diamidino-2-phenylindole staining, anchorage-independent cell transformation assay and western blot analysis. Transfection of exogenous miR-127-3p into MC-3 cells inhibited cell viability and led to $G_1$/S cell cycle arrest. In addition, miR-127-3p also decreased neoplastic cell transformation in TPA-induced JB6 mouse epidermal and MC-3 cells. In addition, miR-127-3p decreased specificity protein 1 (Sp1) expression and increased p21 and p27 expression which are Sp1-dependent cell cycle-related proteins. However, miR-127-3p did not induce apoptosis or affect expression levels of myeloid cell leukemia-1 or survivin. miR-127-3p induced $G_1$/S cell cycle arrest and increased p21 and p27 expression via modulation of Sp1. Therefore, miR-127-3p may be a therapeutic target for human MEC.

## Introduction

Salivary gland tumors are relatively uncommon lesions accounting for 3-6% of all head and neck neoplasms (1) and the annual worldwide incidence is 0.4-13.5 new cases per 100,000 individuals (2-4). Mucoepidermoid carcinoma (MEC) is the most common malignant tumor of the salivary glands, representing 30-40% of all salivary gland malignancies (5). As with the majority of salivary gland tumors, MEC demonstrates morphological diversity and variable biological behavior (6). Therefore, an understanding of carcinogenic mechanisms involved in MEC may offer new target candidates for prognostic and therapeutic strategies (7,8).

microRNAs (miRs) are small endogenous non-coding RNAs that regulate the expression of their target genes at the post-transcriptional level (9). To date, >1,420 miRNAs have been identified in humans (miRBase v17). Each miRNA suppresses multiple mRNA targets (average, ~200) and it is estimated that more than one-third of all human genes are regulated by miRNA molecules (10). Emerging evidence indicates that miRNAs may be associated with the pathogenesis of various types of human cancer (11). The frequent aberrant expression and functional implication of miRNAs in human cancer may be useful as important diagnostic and prognostic biomarkers, as well as potential therapeutic targets (12). However, the importance of miRNA-127-3p in MEC carcinogenesis has not been studied. In the present study, we investigated the growth inhibitory effect of miR-127-3p and the molecular mechanism of this RNA molecule in MC-3 human MEC cells.

## Materials and methods

*Reagents*. FAM-labeled pre-miR negative control #1 (miR-FAM) and hsa-miR-127 pre-miR miRNA precursor (miR-127-3p) were supplied by Ambion (Austin, TX, USA). Antibodies against Sp1, p21, p27 and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). PARP antibody was obtained from BD Pharmingen (San Jose, CA, USA). Antibodies against Mcl-1 and survivin were provided by Cell Signaling Technology, Inc. (Danvers, MA, USA).

*Cell culture and chemical treatment*. MC-3 human MEC cells were provided by Professor Wu Junzheng (Forth Military Medical University, Xi'an, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37˚C in a 5% $CO_2$ incubator. JB6 mouse skin epidermal cells were obtained from the American Tissue Culture Collection (Manassas, VA, USA). Cells were cultured in MEM supplemented with 5% FBS and antibiotics at 37˚C in a 5% $CO_2$ incubator. Cells were transfected with miR-FAM

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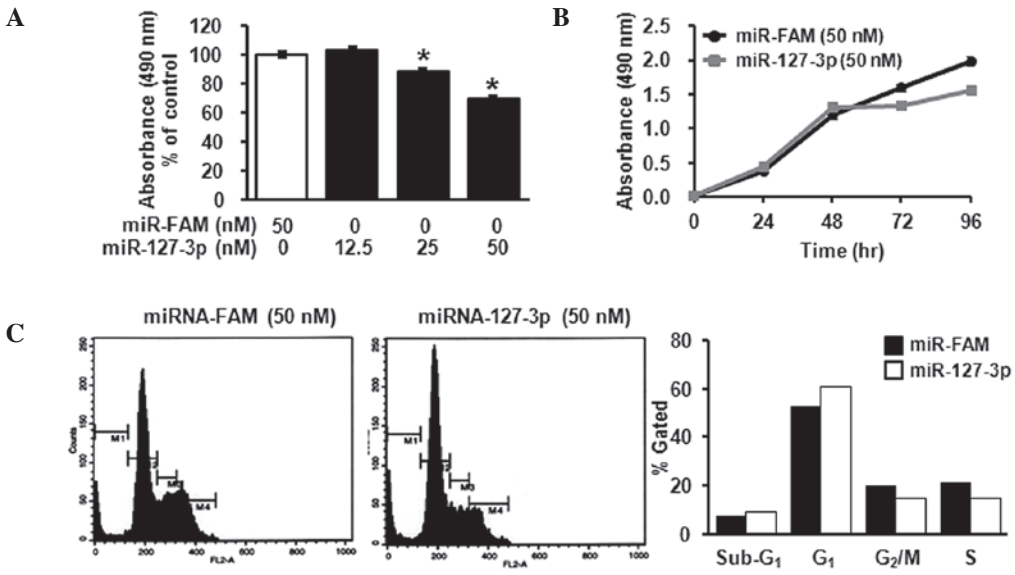


Figure 1. (A) MC-3 cells were transfected with miR-FAM or various concentrations (12.5, 25 and 50 nM) of miR-127-3p for 96 h. The growth inhibitory effect of miR-127-3p was determined using the MTS assay. Graphs present the results of triplicate experiments. *P<0.05, vs. miR-FAM-transfected cells. (B) miR-FAM- or 50 nM miR-127-3p-transfected MC-3 cells were analyzed using the MTS assay at various time-points (24, 48, 72 and 96 h). (C) Cell cycle profiles of MC-3 cells transfected with miR-FAM or 50 nM miR-127-3p were subjected to propidium iodide staining and flow cytometry analysis after 96 h.

or miR-127-3p at various time points (24, 48, 72 or 96 h) and concentrations (12.5, 25 or 50 nM).

*MTS assay*. Effect of miR-127-3p on cell viability was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). MC-3 cells were seeded in 96-well plates and then incubated for 24, 48, 72 or 96 h with various concentrations of miR-127-3p. Absorbance was measured at 490 and 690 nm (background) using an ELISA microplate reader (Bio-Tek Instruments, Inc., Madison, WI, USA).

*Flow cytometry analysis*. Following transfection with miR-FAM or miR-127-3p for 96 h, cells were harvested by trypsinization and fixed overnight in 70% ethanol at -20˚C. The fixed cells were subsequently centrifuged, resuspended in PBS and stained with 0.02 mg/ml propidium iodide. The population of cells in each of the sub-$G_1$, $G_1$, S and $G_2$/M phases was then determined for ~200,000 cells. Cell cycle position was analyzed by FACScan cytometer.

*Anchorage-independent cell transformation assay*. The effect of miR-127-3p on cell transformation was determined in JB6 or MC-3 cells. TPA (20 ng/ml)-stimulated JB6 or MC-3 cells were transfected with miR-FAM or miR-127-3p. Cells were then incubated in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS. Cultures were maintained at 37˚C in a 5% $CO_2$ incubator for 10 days (JB6) or 20 days (MC-3), followed by analysis of colony numbers.

*Western blot analysis*. Whole cell lysates were extracted by lysis buffer and protein concentration of these lysates was quantified using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples containing equal amounts of protein were separated by SDS-PAGE and then transferred to Immun-Blot PVDF membranes (Bio-Rad Laboratories). Membranes were blocked with 5% skimmed milk in TBST at room temperature for 2 h and incubated overnight at 4˚C with primary antibodies against PARP, Sp1, p21, p27, cyclin D1 or actin, followed by incubation with HRP-conjugated secondary antibodies. The antibody-bound proteins were detected using the ECL Western Blotting Luminol reagent (Santa Cruz Biotechnology, Inc.).

*Statistical analysis*. Data are presented as the mean ± SD of triplicate samples from at least 3 independent experiments. Statistical significance was evaluated using a Student's t-test or one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Growth inhibitory effect of miR-127-3p is associated with $G_1$/S arrest*. The effect of miR-127-3p on MC-3 human MEC cells was investigated. Cells were transfected with miR-FAM or various concentrations (12.5, 25 and 50 nM) of miR-127-3p for 96 h and cell viability was examined using the MTS assay. A high concentration of miR-127-3p was identified to significantly decrease cell viability in MC-3 cells (P<0.05), whereas 50 nM of miR-FAM did not change cell viability (Fig. 1A). The time-dependent effect of miR-127-3p on cell viability was also examined. The results demonstrate that 50 nM miR-127-3p decreased cell viability in a time-dependent manner compared with miR-FAM-transfected cells (Fig. 1B). Following this, the growth inhibitory effect of miR-127-3p on cell cycle arrest was analyzed. Fig. 1C demonstrates that 50 nM miR-127-3p caused an elevation in the $G_1$ cell population, indicating that it caused $G_1$/S arrest. These results suggest that the growth inhibitory effect of miR-127-3p is induced by $G_1$/S cell cycle arrest.

*miR-127-3p decreases anchorage-independent colony formation in vitro*. To determine the significance of miR-127-3p in neoplastic cell transformation, JB6 cells with TPA were

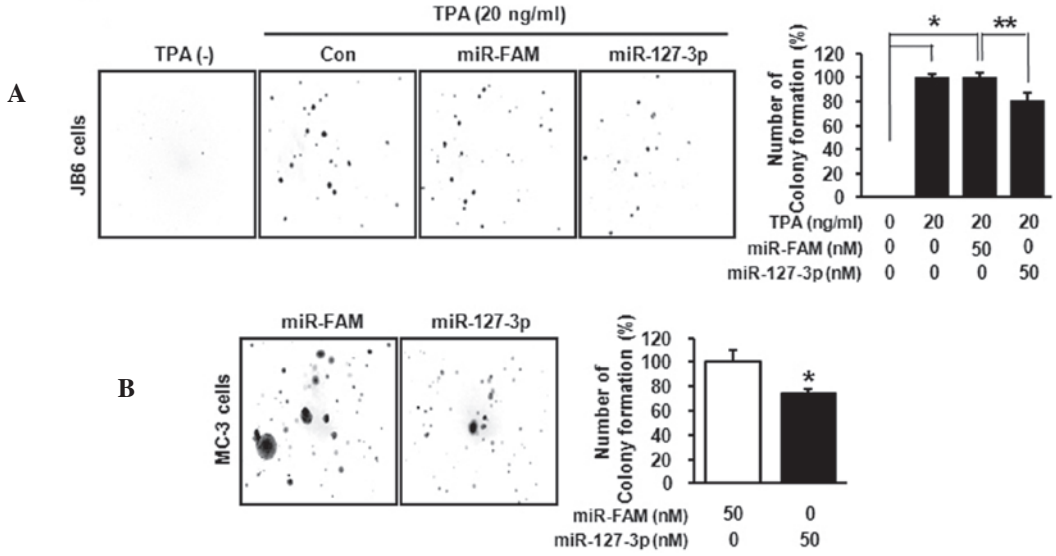


Figure 2. (A) Anchorage-independent cell transformation assay. JB6 mouse skin epidermal cells were stimulated by TPA (20 ng/ml), transfected with miR-FAM or miR-127-3p and cultured in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS for 10 days. Cell colonies were counted. *P<0.05, vs. non-treated cells. **P<0.05, vs. miR-FAM-transfected cells. (B) MC-3 cells transfected with miR-FAM or miR-127-3p were cultured in 1 ml of 0.3% BME agar containing 10% FBS for 20 days and cell colonies were counted. *P<0.05, vs. miR-FAM-transfected cells. Graphs represent the mean of values obtained from the triplicate experiments.

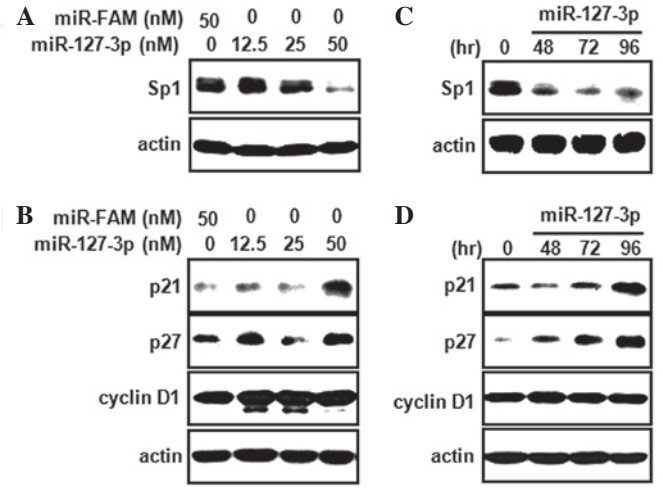


Figure 3. MC-3 cells were transfected with miR-FAM or various concentrations (12.5, 25 and 50 nM) of miR-127-3p for 96 h and whole cell lysates were detected by western blot analysis using antibodies against (A) Sp1, (B) p27, p21 and cyclin D1. miR-FAM- or 50 nM miR-127-3p-transfected MC-3 cells were harvested at various time points (48, 72 and 96 h) and protein levels of (C) Sp1, (D) p27, p21 and cyclin D1 were analyzed by western blot analysis. Equal loading was confirmed using actin protein.

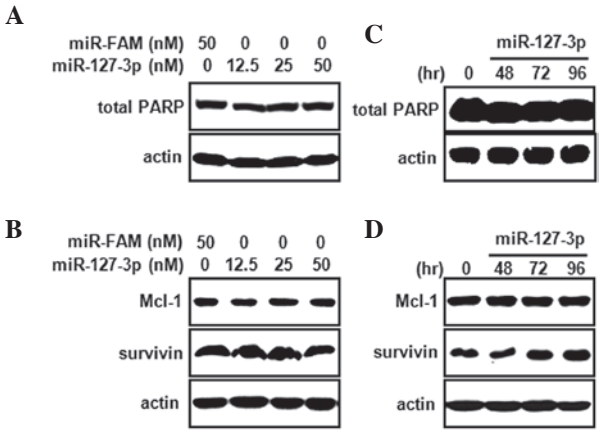


Figure 4. MC-3 cells were transfected with miR-FAM or various concentrations (12.5, 25 and 50 nM) of miR-127-3p for 96 h. Whole cell lysates were extracted and (A) PARP, (B) Mcl-1 and survivin levels were analyzed by western blot analysis. miR-FAM- or 50 nM miR-127-3p-transfected cells were harvested at various time points (48, 72 and 96 h). (C) PARP, (D) Mcl-1 and survivin were detected by western blot analysis.

analyzed using an anchorage-independent colony formation assay (20 ng/ml). miR-127-3p-transfected cells were identified to exhibit significantly fewer and smaller colonies than the miR-FAM-transfected cells (Fig. 2A). In addition, miR-127-3p-transfected MC-3 cells suppressed neoplastic cell transformation (Fig. 2B). These results indicate that miR-127-3p inhibits neoplastic cell transformation *in vitro*.

*miR-127-3p downregulates Sp1 to induce p21 and p27 expression*. Previous studies have demonstrated that several miRNAs regulate Sp1 expression, which is overexpressed in a number of types of tumor and cancer cell lines (13,14). Therefore, we examined whether miR-127-3p regulates the expression of Sp1 protein in MC-3 cells. Fig. 3A and B demonstrate that miR-127-3p induced a concentration- and time-dependent decrease in Sp1 protein. In addition, the correlation between downregulation of Sp1 by miR-127-3p and $G_1$/S arrest-related proteins, including p21, p27 and cyclin D1, was investigated. Fig. 3C and D demonstrate that a significant increase in protein levels of p21 and p27 was identified in cells treated with 50 nM miR-127-3p compared with miR-FAM-transfected cells. This treatment did not affect cyclin D1. These results indicate that the downregulation of Sp1 by miR-127-3p increases p21 and p27 to cause $G_1$/S arrest. Therefore, we hypothesize that $G_1$/S arrest by miR-127-3p in MC-3 cells is associated with an increase of p21 and p27 expression via the modulation of Sp1 protein.

*Growth inhibitory effect of miR-127-3p is not associated with apoptosis*. Next, the growth inhibitory activity of miR-127-3p was investigated to determine the correlation with apoptotic cell death. Fig. 4A and B demonstrate that miR-127-3p did not affect PARP protein levels. Mcl-1 and survivin, the downstream proteins of Sp1, were not altered in miR-127-3p-transfected cells (Fig. 4C and D). These results indicate that miR-127-3p does not inhibit cell growth by a mechanism associated with apoptotic cell death.

## Discussion

miRNAs are endogenous short non-coding RNAs, which contain ~22 nucleotides. A previous study demonstrated that miRNAs regulate the expression of protein-coding genes by translational repression when complementary sequences are present in the 3'-untranslated regions of the target mRNAs or by directing mRNA degradation (15). miRNAs are expressed in a tissue-specific manner and are important for the regulation of a variety of cellular processes, including cell proliferation, apoptosis and differentiation (16-18). In particular, the deregulation of miRNAs is likely to contribute to numerous types of human diseases, including cancer (19). The expression levels of a number of miRNAs have been identified to be significantly decreased in specific types of cancer or cancer cell lines compared with normal tissues (20). Previous studies have identified that miRNAs are downregulated in various cancer cells (21-24). Overexpression of the lethal-7 family of miRNA regulates the expression of proto-oncogenes, including RAS and c-Myc, in cancer tissues (25-27). In addition, restoration of miR-122 in metastatic hepatocellular carcinoma cells was identified to significantly decrease migration, invasion and angiogenesis *in vitro* and *in vivo* (28) and overexpression of miR-330 induced apoptosis through E2F1-mediated suppression of Akt phosphorylation (29). Collectively, these previous studies demonstrate that miRNAs may have tumor suppressor activities in various types of cancer and cancer cell lines through the induction of cell death or apoptosis and regulation of oncogenes.

The aberrant regulation of miR-127, previously identified as miR-127-3p, has been observed in a number of human cancer types (30-32), which indicates tumor-suppressive roles of miRNAs in tumor development. Saito *et al* suggested that miR-127 is constitutively expressed in normal fibroblasts and normal tissues but silenced in cancer cells and downregulated in primary tumors in the prostate and bladder (31). A similar observation was reported by Tsai *et al* (33) identifying that the expression of tumor-suppressive miR-127-3p was significantly downregulated in gastric cancer. In addition, previous studies have revealed a mechanistic link between the downregulation of miR-127 and deregulated apoptosis during carcinogenesis (34,35). These findings indicate the importance of miR-127 in carcinogenesis. In the present study, we identified that the exogenous insertion of miR-127-3p resulted in a marked elevation of the $G_1$ cell population and decreased colony formation in MC-3 human MEC cells, suggesting that miR-127-3p expression may be associated with tumor suppression in MEC, consistent with the results of a previous study (33).

Sp1 protein is a critical transcription factor in tumor development, growth, angiogenesis and metastasis. Moreover, aberrant expression of Sp1 may contribute to cancer development and progression (36) and it is overexpressed in numerous types of human tumor and cancer cells (37,38). A recent study indicated that miR-335 may function as a metastasis suppressor in gastric cancer by targeting Sp1 (14). Previously, our group also reported that Sp1 regulates proteins associated with cell survival (38,39,40). Therefore, we hypothesized that miR-127-3p may regulate the Sp1 protein. Results demonstrate that miR-127-3p decreased Sp1 expression and induced downstream targets, p21 and p27, indicating that the $G_1$/S arrest by miR-127-3p is associated with the modulation of Sp1. Previously, it was revealed that exogenous transfection of miR-29 resulted in loss of mitochondrial potential and release of cytochrome c into the cytoplasm in hepatocellular carcinoma cells, demonstrating that miR-29 induces apoptosis (41). miR-138 also induced apoptosis in head and neck squamous cell carcinoma cells (42). In addition, the downregulation of Bcl-2 by miR-15 and -16 increased activation of the intrinsic apoptosis pathway in leukemic cells (43). These observations illustrate that miRs may affect apoptotic cell death in various types of cancer. Therefore, we investigated whether the exogenous transfection of miR-127-3p induces apoptotic cell death; however, no such effect was observed. Moreover, Mcl-1 and survivin, apoptosis-associated downstream targets of Sp1, were not affected by miR-127-3p. These results indicate that the growth inhibitory effect of miR-127-3p is markedly correlated with $G_1$/S cell cycle arrest and not induction of apoptosis.

In summary, the importance of miR-127-3p in MC-3 MEC cells was investigated. We identified that miR-127-3p mediates antiproliferative activity through the suppression of cell cycle progression via Sp1 protein. Therefore, the present observations indicate that miR-127-3p may be a potential proliferation regulator targeting Sp1 protein against human MEC.

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