Tumor-suppressive Microrna-let-7a Inhibits Cell Proliferation Via Targeting Of E2f2 In Osteosarcoma Cells

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Introduction: MicroRNAs (miRNAs) are evolutionarily conserved, small, non-coding RNA molecules of approximately 22-nucleotides in length that can specifically interact with the 3′-untranslated region (3′UTR) of targeted messenger RNA (mRNA), inhibit mRNA translation, and lead to mRNA cleavage and degradation. miRNAs have the potential to regulate various critical biological processes. The let-7 expression levels were found to be lower in cancer tissues than in the normal lung tissues. However, the role of let-7 in the proliferation of OS cells remains unclear. The E2F family of transcription factors is the downstream effector of the retinoblastoma (Rb) protein pathway. E2F2 plays a central role in the regulation of G1/S transition and cell-cycle progression through the S phase, subsequently promoting the cellular transformation. Although several miRNAs have been found to target E2F2, the correlation of E2F2 expression and miRNA in OS cells is completely unknown. In the present study, we used a genome-wide expression array to analyze both miRNAs and mRNAs in five human OS cell lines and human mesenchymal stem cells (hMSCs). The expression of let-7a was decreased, whereas that of E2F2 was increased in all five OS cell lines compared with the hMSCs. Based on the inverse correlation between let-7a and E2F2 expression, we hypothesize that the effect of E2F2 in OS cells may be mediated, at least in part, through let-7a expression. We aimed to assess whether the expression of E2F2 is regulated by let-7a, and whether the pathway plays a role in the tumorgenesis of OS cells.

Methods: The transfection of let-7a-2-3p mimic or negative control (NC) miRNA and siRNAs was performed using Lipofectamine 2000 (Invitrogen) in antibiotic-free OptiMEM (Invitrogen) according to the manufacturer’s instructions. After 48-h of incubation following transfection, the cells were harvested and processed for further analysis. The cells were plated into 6-well plates (5 x 104 cells/well) and transfected with or without let-7a-2-3p mimic, NC miRNA, or E2F2 siRNA. After 48 h of cultivation, the cells were counted by using the TC10 Automated Cell Counter. To verify the expression of E2F2 protein, antibodies against E2F2 and β-Actin proteins were used and the immunocomplexes were visualized using horseradish peroxidase-conjugated anti-rabbit and the blots were developed by using the ECL Plus System. The quantification of western blot signals was performed by the densitometry using the ImageQuant TL Software (GE Healthcare). All experiments were repeated at least three times. For cell-cycle analysis, the cells were stained with propidium iodide using the CycleTest Plus DNA Reagent Kit (BD Biosciences), and the cell-cycle distribution was analyzed by the FACSVerse Flowcytometer (BD Biosciences). The percentages of cells in the G0/G1, S, and G2/M phases were counted and compared. The quantification of cell death was determined by fluorescence-activated cell sorting (FACS) using the Annexin V-FITC Apoptosis Detection Kit (BD Bioscience). The experimental metastasis model was established by injecting 1 x 106 cells transfected with let-7a miRNA in the gluteal region of nude mice. The mice were divided into three
groups: (1) untreated control (n = 7), (2) transfected with NC-miRNA (n = 7), and (3) transfected with let-7a miRNA mimic (n = 7). The tumor volume of the lung nodule was estimated using the formula “π × long axis × short axis × short axis)/6”.

**Results:** The expression of let-7a decreased by 11.25-21.68-folds in the OS cell lines compared with hMSCs. The cDNA array analysis demonstrated that the expression of E2F2 was increased by 2.36-4.27-fold in all five OS cell lines compared with the hMSCs. The protein expression levels of E2F2 in the let-7a-transfected cells were reduced to 40% of that in the control cells (p < 0.01). Although the expression level of E2F2 protein in the cells transfected with NC siRNA was not significantly affected, the level in the cells transfected with E2F2 siRNA was significantly reduced, as determined by western blotting. The cell growth of MG63 and Saos was inhibited by transfection of let-7a, as determined by cell counting in comparison with untreated and NC-miRNA-transfected cells 48 h after transfection. Like let-7a miRNA-transfected cells, E2F2 siRNA-transfected MG63 and Saos cells showed significant inhibition of cell proliferation compared with untreated and NC siRNA-transfected cells. Both in let-7a- and E2F2 siRNA-transfected cell lines, the number of the cells in the G2/M and G0/G1 phase was significantly lower and higher than that in the untreated or control oligo-transfected cells, respectively. MG-63 cells transfected with let-7a showed statistically smaller tumors in mice than untreated and NC siRNA-transfected cells (Fig. 6d), indicating that let-7a also inhibits the growth of OS cells in vivo.

**Discussion:** The biological roles of let-7a in OS cells have not yet been clarified. Our results indicated that the expression of let-7a was coordinately upregulated in the OS cell lines, which led to us to performing genome-wide mRNA profiling by cDNA array to detect the possible targets of let-7a in the OS cells. Although let-7a probably influences the expression of several genes, we focused on E2F2 as the target of let-7a in OS cells. Our cDNA array analysis demonstrated that E2F2 was the only let-7a target gene whose expression was uniformly upregulated in all five OS cell lines, whereas the expression of other candidate genes differed among the OS cell lines. Thus, we analyzed the possibility that let-7a may contribute to anticancer activities by targeting E2F2 in the OS cells.

We next examined the functions of let-7a in the regulation of its possible target gene, E2F2, and the changes in the biological characteristics in the OS cell lines. The forced elevation of let-7a levels resulted in the reduction of the expression of E2F2 protein, indicating that let-7a might function as a tumor suppressor gene in the OS cells.

Our data regarding the cell cycle showed that let-7a inhibited the proliferation of OS cells via induction of the cell-cycle arrest at the G1/G0 phase. We can assume that the upregulation of let-7a might affect the cell-cycle progression of OS cells via let-7a-mediated control of the E2F2 expression. Notably, the downregulation of E2F2 by challenge of let-7a miRNA or siRNA against E2F2 did not induce apoptosis of OS cells, indicating that the repression of OS cell growth was acquired by cell-cycle retardants. Furthermore, the overexpression of let-7a in the OS cells resulted in the inhibition of OS tumor growth in vivo. In concordance with the data of in vitro experiments, the xenograft model of OS suggested that let-7a induction could inhibit OS cells development in vivo by targeting E2F2 expression.

**Significance:** Our data suggest that E2F2 is one of the crucial factors that enhances tumor proliferation in OS, as other malignant tumors. Although the data presented in this study needs to be confirmed by using clinical OS samples, information regarding the association between let-7a and E2F2 in OS cells would be beneficial for determining the underlying mechanisms of OS and may facilitate the development of novel therapeutic strategies for clinical application.