MS275 up-regulates Fas expression through down-regulation of miR-20a in osteosarcoma cells

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Abstract

Despite aggressive chemotherapy treatments, prognosis for patients with lung metastases from osteosarcoma (OS) remains poor. It is thus important to pursue new therapeutic approaches. The ability of osteosarcoma cells to form lung metastases has been inversely correlated to cell surface Fas expression. Down-regulation of Fas allows OS cells to avoid FasL-mediated apoptosis within the FasL positive lung microenvironment. The histone deacetylase inhibitor, MS275, has been shown to up-regulate Fas expression in Fas negative LM7 OS cells and induce regression of established OS lung metastases. But the mechanism through which MS275 exerts its effect is unknown.

Recently, studies have shown that overexpression of miR-20a, which is part of the miR-17-92 cluster, results in the down-regulation of Fas expression in SAOS-2 cells and a decreased sensitivity to FasL. Thus, a potential mechanism through which MS275 up-regulates Fas expression is through the regulation of miR-17-92 and miR-20a. In the current study, LM7 cells were treated with MS275 and its effects on miRNA expression were studied. To address the underlying mechanism of MS275, we characterized the effect of MS275 on miRNA gene transcription and degradation. MS275 suppressed miR-17-92 and miR-20 levels in a time dependent manner. Although MS275 decreased miRNA expression, it enhanced the promoter activity for miR-17-92 as measured by luciferase assay, suggesting that the decrease in miRNA following MS275 treatment is mediated through a post-transcriptional mechanism. Experiments using actinomycin D, a DNA transcription inhibitor, and cycloheximide, a protein translation inhibitor, demonstrated that MS275 did not function by enhancing post-transcriptional degradation. This data suggests that down-regulation of miR-20a by MS275 may occur through other factors that remain to be described.

Introduction

OS is the most common pediatric primary bone tumor that metastasizes almost exclusively to the lung. Despite aggressive chemotherapy treatment, prognosis of metastatic OS remains poor, indicating the need for new therapies. FasL has been shown to be constitutively expressed in the lung, where Fas positive cells are eliminated. OS lung metastases from patients have been shown to be Fas negative[1]. In addition, the metastatic potential of OS cells was shown to be inversely correlated to cell surface Fas expression[2]. Recently, histone deacetylase inhibitors (HDACi) have emerged as a promising new class of anticancer drugs. HDACi allow for histones to remain acetylated and in an open conformation in which they are transcriptionally activated. MS275 (Fig. 1), a class I HDACi, has been shown to up-regulate Fas expression in human Fas negative LM7 OS cells and induce regression of established OS lung metastases[3]. Yet the mechanism by which MS275 upregulates Fas is unknown.

Recently, a specific miRNA (miR-20a) has been shown to down-regulate Fas and be responsible for the low Fas expression in OS cells[4]. miR-20a, which is part of the miR-17-92 (Fig. 1) cluster down-regulated Fas expression in non-metastatic Fas positive SAOS-2 OS cells following transfection. Inhibition of miR-20a in Fas negative metastatic LM7 cells (derived from SAOS-2) resulted in a decrease in their metastatic potential. We hypothesize that MS275 up-regulates Fas expression through the down-regulation of miR-17-92 and miR-20a. We also aim towards characterizing the underlying mechanism through which MS275 exerts its action.

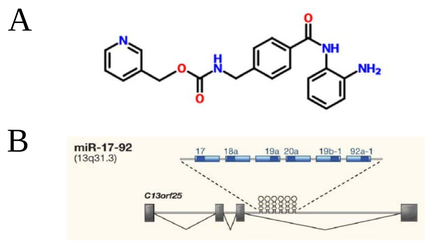

Fig 1. Chemical structure of MS-275 (A) and schematic representation of the miR-17-92 cluster (B).

Methods

The metastatic LM7 cell line was developed by repetitive cycling of SAOS-2 cells through the lungs of nude mice 7 times[5]. Cell lysates were processed by Western blot analysis as described previously[6]. Total RNA was isolated and purified from cultured cells by the RNeasy Mini Kit (Qiagen, Inc). A regular reverse transcription was carried out using the Reverse Transcription System with oligo-dT primer (Promega Corporation) according to the manufacturer's instructions. The resulting DNA was used for PCR amplification with Taq-polymerase (Promega). mir-17-92 promoter luciferase construct (pro 1353) was obtained from Dr. Scott M. Hammond[7]. For the promoter activity assay, LM7 cells were transfected with luciferase reporter construct for 6 hours, followed by MS-275 treatment. Luciferase activity was measured after 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega). The unpaired Student t test was used to determine the significance of differences between experimental groups. P values <.05 were considered statistically significant.

Results

Effect of Fas protein expression by MS275

Fas negative LM7 were selected and cloned from the LM7-J99 cell line. Two separate cell lines were created in this way, LM7_C2 and LM7_C15. Treatment of these cells with MS275 (2uM) resulted in significant increase of Fas protein after 48 hours (Fig. 2).

Results (continued)

MS275 down-regulates miR-17-92 and miR-20a expression

LM7 cells were treated with MS275 (2uM) or EtoH (control) for 3, 6, and 24 hours to measure miR-17-92 expression and 3, 6, 24, and 48 hours to measure miR-20a expression. miRNA levels were measured using real time PCR. miR-17-92 expression decreased significantly by 24 hours (Fig. 3A), and 48 hours for miR-20a (Fig. 3B)

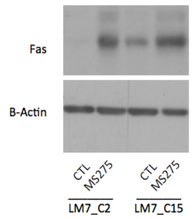

Fig 2. MS-275 (2uM) up-regulates Fas protein levels as measured by western blot in Fas negative LM7 C2 and C15 cell lines after 48 hours.

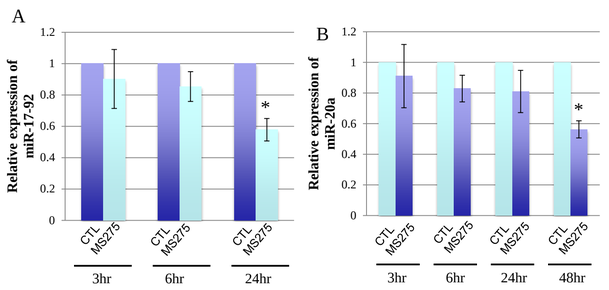

Fig 3. (A) miR-17-92 cluster and (B) miR-20a is down-regulated after treatment with MS275.

MS275 up-regulates the promoter activity of the miR-17-92 cluster

In detailing the mechanism of action of MS275 in regulation of miR-17-92 and miR-20a expression, we first studied the promoter activity of the miR-17-92 cluster using a luciferase assay in LM7 cells. Paradoxically, MS275 induced an upregulation of miR-17-92 promoter activity (Fig 4) while exerting a down-regulatory effect on miRNA levels.

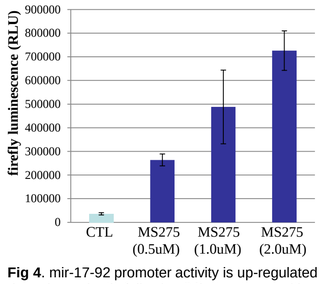

Fig 4. mir-17-92 promoter activity is up-regulated dose dependently following 24hr treatment with MS275 in LM7.

MS275 may not enhance down-regulation of miR-20a

Another mechanism by which down-regulation of miR-20a may occur is through transcriptional induction of a gene involved in its degradation. Actinomycin D, a transcription inhibitor, was used in combination with MS275 . In doing so, miR-20a expression levels remained comparatively the same to MS275 untreated cells (Fig. 5A), demonstrating that MS275 did not induce increased degradation of miR-20a. This may suggest that new protein synthesis is required for MS275 induced decrease of miR-20a. In order to test this, we measured expression levels of miR-20a in the presence of cycloheximide, a protein synthesis inhibitor, and MS275. However, there was no difference in miR-20a levels between cells treated with MS275 alone and those treated with MS275 and CHX together (Fig. 5B), suggesting that protein translation may not be necessary for miR-20a degradation.

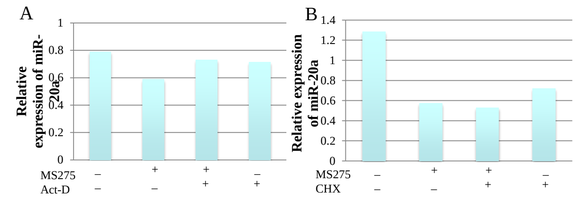

Fig 5. (A) Real time PCR analysis of miR-20a expression with MS275 (2uM, 6 hours), in the absence and presence of actinomycin D (Act-D, 2.0 ug/mL). (B) real time PCR analysis of miR-20a expression with MS275 (2uM, 48 hours), in the absence and presence of Cycloheximide (CHX, 40 ug/mL).

Conclusions

Fas expression, regulated by miR-17-92 and miR-20a levels, correlates inversely with the metastatic potential of osteosarcoma (OS) cells. MS275 downregulates miR-17-92 and miR-20a expression, resulting in an increase in Fas protein levels. This down-regulation of miRNA induced by MS275 is not mediated by an effect on the promoter activity or the degradation of the miRNA. MS275 may be involved in expression of other factors that regulate miRNA processing, miRNA intrinsic stability, or protection and degradation methods related to target RNAs.

References

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