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THE microRNA-29 PLAYS A CENTRAL ROLE IN OSTEOSARCOMA PATHOGENESIS AND PROGRESSION

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Osteosarcoma is the most common type of bone cancer, with a peak incidence in the early childhood. The relationship between microRNAs (miRNAs) and cancer development attracted more and more attention over the last few years. Members of the miRNA-29 family, including miRNA-29a, miRNA-29b, and miRNA-29c were shown to participate in the development of rhabdomyosarcoma and hepatocarcinogenesis. Here, it has been demonstrated miRNA-29a and miRNA-29b expression levels to be downregulated in most of the osteosarcoma tissues (23 from 30). Besides, miRNA-29a displayed ability to induce apoptosis in both U2OS and SAOS-2 osteoblastic cells. While miRNA-29 members induced apoptosis through *p53* gene activation, the effect of miRNA-29a on osteoblastic cells was independent on *p53* expression level. Moreover, *Bcl-2* and *Mcl-1* were earlier demonstrated to be the direct targets of miRNA-29 in many types of cancer tissues and cancers. In both U2OS and SAOS-2 osteoblastic cell types, overexpression of miRNA-29a also downregulated *Bcl-2* and *Mcl-1*, while silencing of miRNA-29a increased their expression. In addition, enhanced expression of miRNA-29a increased the expression of two tumor suppressor genes, *E2F1* and *E2F3*. In summary, data obtained highlight the role of miRNA-29a in the regulation of osteoblastic cell apoptosis by silencing *Bcl-2* and *Mcl-1* and inducing *E2F1* and *E2F3* expression.

Keywords: **miRNA-29, osteosarcoma, apoptosis, cell cycle, U2OS cells, SAOS-2-2 cells.**

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional and/or translational levels [1]. miRNAs play an important role in various biological processes, including development, cell proliferation, differentiation and metabolism [2, 3]. Recent studies have proved that expression of many miRNAs is altered in various cancers, suggesting that miRNAs play a key role in carcinogenesis [4, 5], and particularly miRNA-29 is an important factor in development of hepatocellular cancer, acute myeloid leukaemia and rhabdomyosarcoma [6, 7].

Osteosarcoma is the most common type of bone cancer, with a peak incidence in early childhood [8]. It is associated with a poor prognosis that is resulted from its resistance to chemotherapy, and propensity to metastasise to the lungs [8, 9]. Osteosarcoma is an aggressive cancerous neoplasm arising from primitive transformed cells of mesenchymal origin that exhibit osteoblastic differentiation and produce malignant osteoid tissue. Osteoblasts are derived from mesenchymal stem cells, and osteoblastic differentiation is tightly regulated by numerous growth and differentiation factors, such as bone morphogenetic proteins and Notch signalling [10]. It is conceivable that any disruption of the osteogenic terminal differentiation may result in the development of osteosarcoma.

Downregulation of miRNA-29 family members, including miRNA-29a, miRNA-29b and miRNA-29c, was observed in other types of human neoplasms [11–15]. It was shown that overexpression of miRNAs-29 inhibited tumour growth in rhabdomyosarcoma mouse models [16] and ectopic expression of miRNA-29b promoted tumour necrosis factor-related apoptosis, inducing ligand-triggered apoptosis [14]. In addition, miRNA-29a, miRNA-29b and miRNA-29c significantly repressed lung cancer viability *in vivo* [17]. To date, several oncogenes, including *T-cell leukaemia/lymphoma 1* (*TCL1*), *cell division cycle 42* (*CDC42*), *Ying Yang 1* and *phosphoinositide-3-kinase*, were shown to be of miRNA-29a, miRNA-29b and miRNA-29c targets [16, 18]. However, the role of the miRNA-29 in osteosarcoma and the signalling pathway by which miRNA-29 exerts its function in osteosarcoma cells remains largely unexplored.

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Here, it has been shown that the miRNA-29 expression markedly reduced in the majority of examined osteosarcoma tissues. Furthermore, the function of the miRNA-29 in two osteosarcoma cell lines was intensively investigated.

EXPERIMENTAL

Tissue collection. Surgically resected osteosarcoma tumour tissues and adjacent normal tissues were collected from 30 primary osteosarcoma cancer patients. The study was approved by the Fudan Hospital ethical committee. Participating individuals provided written informed consent. These investigations were conducted according to the principles of the Declaration of Helsinki.

Reverse transcription polymerase chain reaction (RT-PCR) and real time PCR analysis. Total RNA was isolated using the mirVana miRNA isolation kit ("Applied Biosystems", USA). TaqMan Reverse Transcription Kit ("Applied Biosystems") was used for cDNA synthesis according to the manufacturer's protocol. miRNA-29a, miRNA-29b, miRNA-29c, and the endogenous control were purchased from ("ABI", USA). A two-step RT-PCR, with reverse transcription using a miRNA-specific primer, followed by quantitative PCR with TaqMan probes, was carried out according to the manufacturer's instructions.

Data from three independent experiments were analysed using the Student's t-test, and $p < 0.05$ was considered to be statistically significant.

Reagents, cell lines, cell transfection. Human osteosarcoma cell lines U2OS and SAOS-2 were cultured in DMEM ("Invitrogen") supplemented with 10% fetal bovine serum ("Invitrogen"). All antibodies used in this study were purchased from "Santa Cruz". miRNA-29 mimics, antagonists and the control miRNA mimic were obtained from "Thermo Scientific Dharmacon". Osteosarcoma cells were transfected 24 h after being seeded in 6-well plates using the Lipofectamine2000 transfection reagent ("Invitrogen").

Cell viability and apoptosis analysis. Osteosarcoma cells were transfected with miRNA-29 mimics or the control mimic. After an additional incubation for 72 h, the cells were harvested, stained with propidium iodide and FITC-conjugated anti-(annexin V)-antibodies and analysed using FACS.

Luciferase reporter assay. The 3'-UTR region of *E2F1*, *Bcl2* or *Mcl1* gene, which contains the putative miRNA-29 binding sequence, was cloned into a luciferase reporter construct (pMIR-Report plasmid; "Ambion"), respectively. Luciferase activity was measured 24 h after transfection. Experiments were repeated three times independently. The primers for the construction of these three plasmids are listed below:

E2F1:

F: 5'-GCGTGTAGGACGGTGAGA-3';

R: 5'-GGAGAAGGGAAGTGGAGAAT-3'.

Bcl2:

F: 5'-TAAGACAGTCCCATCAAA-3',

R: 5'-AATAGTGTATAGGCCATAAC-3'.

Mcl1:

F: 5'-CAAGTGGCAAGAGGATTA-3',

R: 5'-TCTGTAGAGGGAGCAGAA-3'.

Statistical analysis. The data are expressed as the mean ± standard error of the mean from at least three independent experiments. Unless otherwise noted, the differences between groups were analysed using the Student's t-test for two-group comparisons or by one-way analysis of variance when more than two groups were compared.

RESULTS

Downregulation of miRNA-29 is a frequent event in osteosarcoma tissues

First, the miRNA-29 expression in 30 paired osteosarcoma and adjacent normal tissues using real-time PCR was analysed. It was found that miRNA-29a, miRNA-29b and miRNA-29c were significantly downregulated in most of the osteosarcoma tissues (23 from 30), especially miRNA-29a and miR-29b (fig. 1*a*). Considering that some miRNAs, such as miRNA-21, miRNA-34 and miRNA-143 are regulated by p53, we next investigated whether the miRNA-29 downregulation was correlated with p53 expression. Using two osteosarcoma cell lines, U2OS ($p53^{+/+}$) and SAOS-2-2 ($p53^{-/-}$), to examine the expression of miRNAs-29, we have found that both cell lines expressed miRNAs-29 at similar levels (fig. 1*b*), suggesting that the basal miRNAs-29 levels in these cells are p53-independent. Furthermore, U2OS cells were treated with adriamycin, and the expression of miRNAs-29 was measured. Although p53 can be readily induced in cells by exposure to genotoxic stress (data not shown), expression levels of miRNAs-29 were not changed in U2OS cells (fig. 1*c*).

miRNA-29 inhibits cell proliferation and promotes cell apoptosis

The downregulation of the miRNA-29 in osteosarcoma tissues prompted us to investigate its biological functions in carcinogenesis. Aberrant cell proliferation is a hallmark of cancers. A significant decrease ($p < 0.05$) in proliferation after transfection of miRNA-29 in U2OS cells was observed (fig. 2*a*,*b*). In contrast, transfection of the miRNA29a antagonist significantly ($p < 0.05$) increased the cell proliferation ratio (fig. 2*b*). This indicated that cell proliferation was significantly affected by an increase in miRNA-29 expression. Further, the effect of miRNA-29 on apoptosis was investigated, and it was found a dramatic increase in apoptosis (fig. 2*c*) in U2OS and SAOS-2 cells 72 h after transfection with the miRNA-29a mimic,

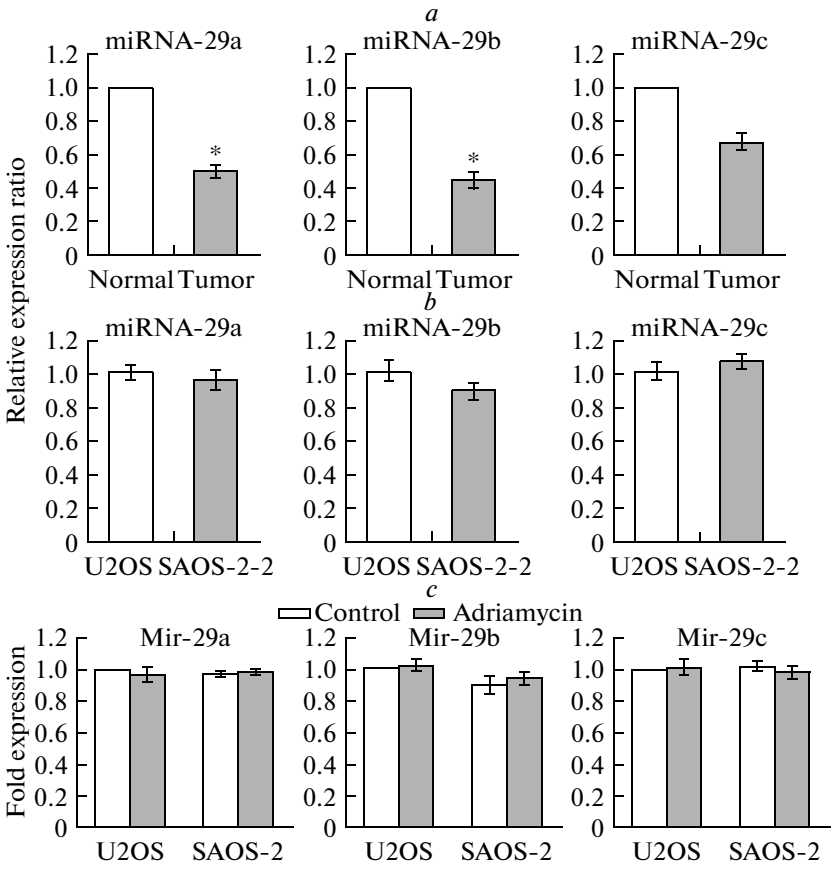

Fig. 1. A family of miRNA-29 is frequently downregulated in osteosarcoma tissues. Total RNA was extracted from 30 paired osteosarcoma and adjacent normal tissues, U2OS or SAOS-2 cells. Expression of miRNAs-29 was measured by real-time PCR. The relative expression levels of miRNAs-29 in adjacent normal tissues or U2OS cells were set as 1. *a* – Comparison of expression of miRNAs-29 in osteosarcoma and adjacent normal tissues. *b* – The expression of miRNAs-29 in untreated U2OS and SAOS-2 cells. *c* – The expression of miRNAs-29 in 200 nM adriamycin-treated or untreated U2OS and SAOS-2 cells. The data are presented as the mean ± SD. Asterisk: $p < 0.05$ versus normal.

suggesting that miR-29 may function as a strong apoptotic factor in osteosarcoma cells.

Overexpression of miRNA-29 decreases Bcl-2 and Mcl-1 protein levels

Next, molecular mechanisms of miRNA-29 were investigated. *Bcl-2* and *Mcl-1* were demonstrated [14, 27] to be direct targets of miRNA-29 in many types of cancer tissues and cancer cell lines. To verify, whether *Bcl-2* and *Mcl-1* are also targets of miRNA-29 in osteosarcoma, we employed a dual-luciferase reporter assay. Co-transfection of miRNA-29a significantly suppressed the 3'-UTR reporter of *Bcl-2* and *Mcl-1* in U2OS and SAOS-2 cells (fig. 3*a,b*). Moreover, an overexpression of miRNA-29a led to a decrease in levels of endogenous Bcl-2 and Mcl-1 (fig. 3*c*), while transfection of the miRNA-29a antagonist increased the protein levels (fig. 3*d*). These results clearly demonstrated that the miRNA-29 level inversely correlated with Bcl-2 and Mcl-1 expression in osteosarcoma cells.

miRNA-29 affects expression of target genes related to the cell cycle arrest

Further, to elucidate the role of miRNA-29, gene expression in U2OS cells after transfection with the miRNA-29a mimic was examined. It was found both *E2F1* and *E2F3* being significantly upregulated, *cyclin D1* and *cyclin E2* being moderately downregulated (fig. 4*a*), and *CDK6* and *CDK7* levels being not affected. To prove that these changes were direct effects of miRNA-29, we fused the 3'-UTRs of *E2F1* to a luciferase reporter construct. The transfection of U2OS cells with the miRNA-29 mimic effectively increased luciferase reporter gene expression (fig. 4*b*). Similar results were obtained for SAOS-2 cells (data are not shown).

DISCUSSION

Although misexpression of miRNAs was observed in various types of cancers, the molecular mechanisms by which miRNAs modulate the process of carcinogenesis and the behaviour of cancer cells remain large-

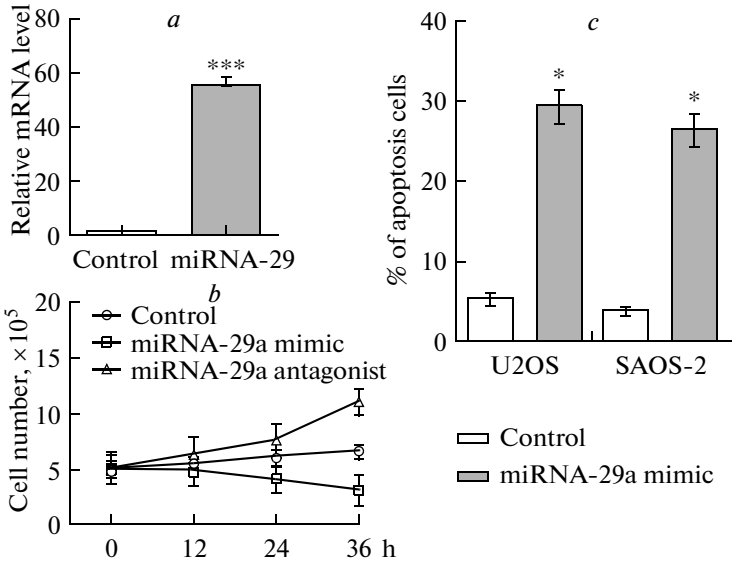

Fig. 2. Members of miRNA-29 family inhibit cell proliferation and promote cell apoptosis. *a* – Overexpression of miRNAs-29 in U2OS cells as determined by real-time PCR. *b* – U2OS cells were transfected with miRNA-29 mimics, or a miRNA-29 antagonist, or the control mimic for the indicated time, and number of cells was determined by trypan-blue staining. *c* – U2OS and SAOS-2 cells were transfected with either miRNA-29 mimics or the control mimic for 72 h; cells were trypsinized, washed with PBS, fixed, stained with propidium iodide, FITC-conjugated with anti-(annexin V)-antibodies and subjected to FACS analysis. The percentage of apoptotic cells is presented as the mean ± SD from three independent experiments. Asterisk: $p < 0.05$ versus control.

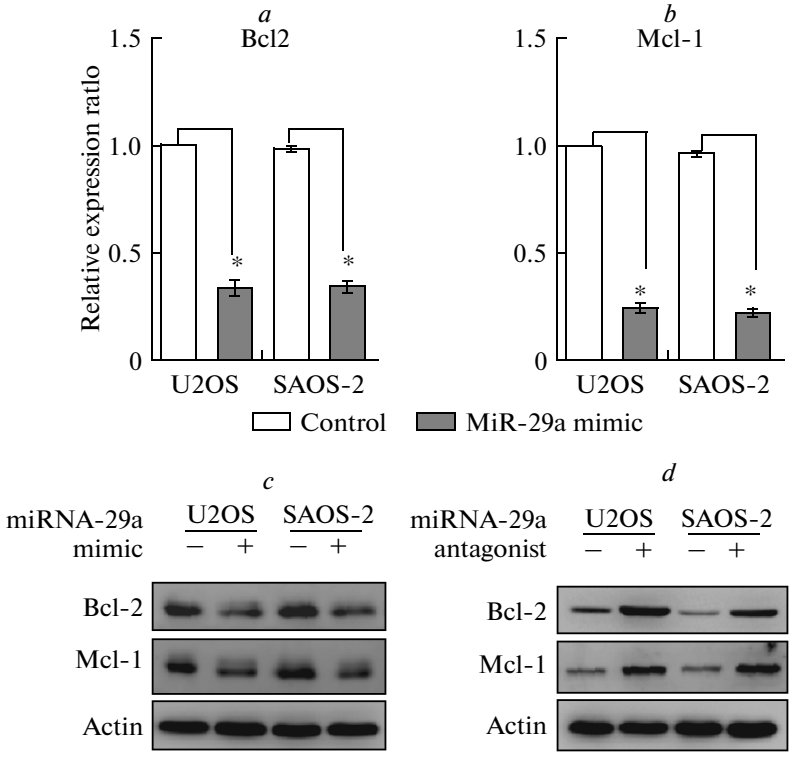

Fig. 3. Overexpression of miRNAs-29 decreases Bcl-2 and Mcl-1 protein levels. *a*, *b* – U2OS and SAOS-2 cells were co-transfected with luciferase reporter constructs together with miRNA-29a mimic or control mimic as indicated, and harvested 24 h later. A luciferase assay was performed, and the relative luciferase activity in control U2OS cells was set as 1. The data are presented as the mean ± SD from three independent experiments. Asterisk: $p < 0.05$ versus control. *c, d* – U2OS and SAOS-2 cells were transfected with a miRNA-29a mimic or antagonist, or control mimic as indicated, and harvested 24 h later. Protein expression was detected by Western blot using the indicated antibodies.

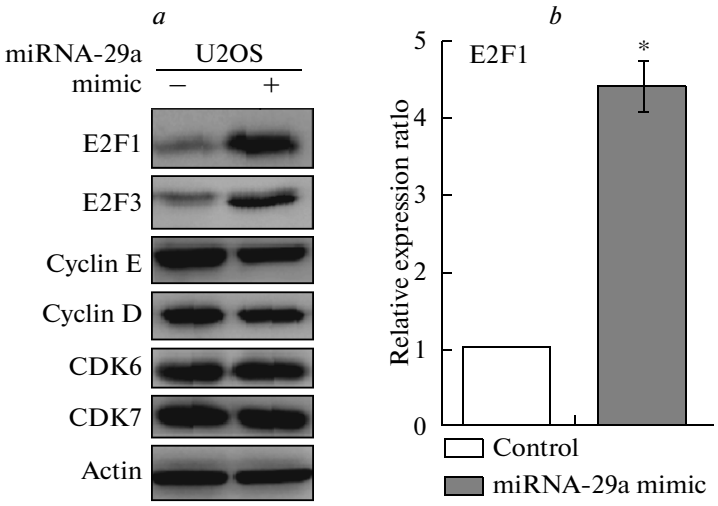

Fig. 4. The miRNA-29 affects the expression of target genes related to cell cycle arrest. *a* – U2OS cells were transfected with miRNA-29 mimics or the control mimic as indicated, and harvested 24 h later. Protein expression was detected by Western blot using the indicated antibodies. *b* – U2OS cells were co-transfected with luciferase reporter constructs and either a miRNA-29a mimic or control mimic as indicated, and harvested 24 h later. A luciferase assay was performed, and the relative luciferase activity in the control U2OS cells was set as 1. The data are presented as the mean ± SD from three independent experiments. Asterisk: $p < 0.05$ versus control.

ly unknown[19, 20]. Here, it has been demonstrated that downregulation of miRNA-29 is a frequent event in osteosarcoma tissues and forced expression of miRNA-29 in osteosarcoma cells inhibits cell proliferation and promotes cell apoptosis. Further, Bcl-2 and Mcl-1 have been characterised as functional targets of miRNA-29. Data obtained suggest a fundamental role of miRNA-29 in tumour genesis, as well as in the phenotypes of cancer cells, and implicate the potential application of miRNA-29 in prognosis prediction and cancer therapy.

Both Bcl-2 and Mcl-1exert an antiapoptotic function through the mitochondrial signalling pathway [21]. It was_shown that *Mcl-1* and/or *Bcl-2* gene expression is upregulated in different types of cancers, and their overexpression is correlated with tumour progression and poor prognosis [22, 23] that is consistent with results obtained here. These data suggest that the functional loss of the miRNA-29 family may result in enhanced expression of *Bcl-2* and *Mcl-1* and, in turn, resistance of cells to apoptosis, which consequently favours tumour progression.

In addition, two tumour suppressor genes, *E2F1* and *E2F3*, were identified as targets of miRNA-29 in osteosarcoma cells. The *E2F* is a group of genes that codifies a family of transcription factors (TF) in higher eukaryotes [24, 25]. All of these genes are involved in the cell cycle regulation and synthesis of DNA in mammalian cells [26]. Results obtained suggest that the *E2F1* and *E2F3* expression has a positive correlation with the expression of miRNA-29 in osteosarcoma cell lines at the transcriptional and translational levels. Thus, the miRNA-29-mediated network might be much more complex than previously thought, and therefore, identifying all of the important targets and understanding the relevant molecular pathways in various physiological and pathologic conditions will be very important to completely understand biological functions of these miRNAs.

In summary, we investigated the potential role of the miRNA-29 family in tumour genesis and its underlying mechanisms. Data obtained suggest that downregulation of miRNA-29 may play an important role in the development of cancer, such as osteosarcoma, and miRNA-29 may be employed as a prognosis marker and therapeutic target for osteosarcoma.

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