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MiR-204/14-3-3ζ axis regulates osteosarcoma cell proliferation through SATA3 pathway

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Hyperproliferation of cells is a major problem is osteosarcoma (OS). So, further elucidation of the molecular mechanisms underlying hyperproliferation of OS is needed. Western blots results showed that 14-3-3ζ protein was upregulated in OS cell lines; 14-3-3ζ knockdown significantly suppressed OS cell proliferation, as well as the protein levels of p-STAT3, c-Myc and Cyclin D1. MicroRNA-204 (miR-204) has been regarded as an essential regulator in cancer carcinogenesis, including OS. Here, we revealed that miR-204 directly targets the 3'UTR of 14-3-3ζ to inhibit its expression, thus to suppress 14-3-3ζ-induced OS cell hyperproliferation. Further, we demonstrated that the STAT3 pathway was involved in miR-204/14-3-3ζ regulation of OS cell proliferation. Our findings provide information about the underlying mechanisms of miR-204/14-3-3ζ in OS cell proliferation through the STAT3 pathway, and suggest miR-204 and 14-3-3ζ as potential therapeutic targets in OS.

1. Introduction

Osteosarcoma (OS), the most common mesenchymal sarcoma in bones, mainly arises from the metaphysis of the long bones of adolescents and young adults (Thompson 2013). Despite the fact that great efforts in the early diagnosis and effective treatment of OS have been achieved, the 5-year survival rate of patients with recurrent or metastatic OS remains at ~30% (Thompson 2013). As the deregulation of oncogenes or tumor suppressors has been found to play key roles in the growth and metastasis of OS, development of potential molecular targets holds promise for the effective therapy of OS (Liang et al. 2013).

14-3-3 proteins are a family of conserved regulatory molecules that are expressed in all eukaryotic cells. 14-3-3 proteins have the ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors. More than 200 signaling proteins have been reported as 14-3-3 ligands. 14-3-3ζ, encoded by the *YWHAZ* gene on chromosome 8 (Tommerup and Leffers 1996), is a major regulator of apoptotic pathways critical to cell survival and plays a key role in a number of cancers and neurodegenerative diseases (Liang et al. 2014; Matta et al. 2012; Nishimura et al. 2013; Weerasekara et al. 2014).

The STAT proteins are a conserved family of transcription factors implicated in regulating processes such as inflammation, survival, proliferation, metastasis, angiogenesis, and chemoresistance of tumor cells (Levy and Darnell 2002). One of these members, namely STAT3, is ubiquitously expressed and is functionally involved in regulating cell proliferation, differentiation and cell survival (Catlett-Falcone et al. 1999; Levy and Lee 2002). In many cancer cells, STAT3 signaling has been recognized as a pivotal pathway supporting survival and growth (Catlett-Falcone et al., 1999; Grandis et al. 2000; Mora et al. 2002). STAT3 activation occurs when the tyrosine 705 (Tyr705) or serine 727 (Ser727) residues are phosphorylated (Bowman et al. 2000; Breit et al. 2015; Faruqi et al. 2001; Kaptein et al. 1996; Murase and McKay 2014; Zhang et al. 2013), leading to STAT3 binding to target genes induces the transcription and upregulation of proliferation and antiapoptotic associated proteins (Bowman et al. 2000; Bromberg et al. 1999; Real et al. 2002).

Interestingly, 14-3-3ζ has been reported to interact with STAT3 and regulates its constitutive activation in multiple myeloma cells (Zhang et al. 2012). During the angiogenesis in non-small cell lung cancer (NSCLC), 14-3-3ζ enhanced the phosphorylation of STAT3, thus to initiate the STAT3/HIF-1α/VEGF feed-back loop to promote the angiogenesis in NSCLC (Xue et al. 2016). However, the functions of 14-3-3ζ in OS, whether 14-3-3ζ regulate STAT3 to exert its function in OS and the mechanism(s) still remain unclear.

The miRNAs are a group of small non-coding RNAs, which inhibit the target genes by binding to their 3' UTR (Kobayashi et al. 2012; Maire et al. 2011; Ram Kumar et al. 2016). MiR-204 is an important tumor suppressor in cancers, including OS (Li et al. 2016; Shi et al. 2015; Zhang et al. 2016). It inhibits proliferation, migration, invasion and epithelial-mesenchymal transition in osteosarcoma cells via targeting Sirtuin 1 (Shi et al. 2015).

Given the essential roles of 14-3-3ζ and STAT3 pathway in regulation of cell proliferation; here we investigated the detailed functions of 14-3-3ζ in OS cell proliferation. Further, we evaluated the protein levels of STAT3 pathway-related factors to validate whether STAT3 pathway was involved in 14-3-3ζ regulation of OS proliferation. The interaction between miR-204 and 14-3-3ζ was later validated. Finally we investigated whether miR-204/14-3-3ζ modulated OS cell proliferation through STAT3 pathway. Our present study aimed to indicate the potential signal transduction initiated by miR-204/14-3-3ζ, and to provide a better understanding of miR-204- and/or 14-3-3ζ-caused hyperproliferation of OS cell.

2. Investigations and results

2.1. Expression of 14-3-3ζ in OS cell lines and its promotive effect on OS cell proliferation

Firstly, we evaluated the protein expression levels of 14-3-3ζ in four OS cell lines, 143B, Saos2, MG63 and U2OS, using Western blot. As exhibited in Fig. 1A, 14-3-3ζ expression was significantly upregulated in all four OS cell lines, and more strongly upregulated in MG63 and Saos2 cells. These two cell lines were chosen as further cell models. Next, we achieved 14-3-3ζ knockdown by transfecting si-14-3-3ζ into MG63 and Saos2 cells, as verified using Western blot (Fig. 1B). Then the cell viability and colony formation capability was determined using MTT and colony formation assays. Results showed that, after 14-3-3ζ knockdown, the cell viability and colony formation n capability of both MG63 and Saos2 cell lines were significantly suppressed (Fig. 1C-F), indicating the promotive role of 14-3-3ζ in regulation of OS cell proliferation.

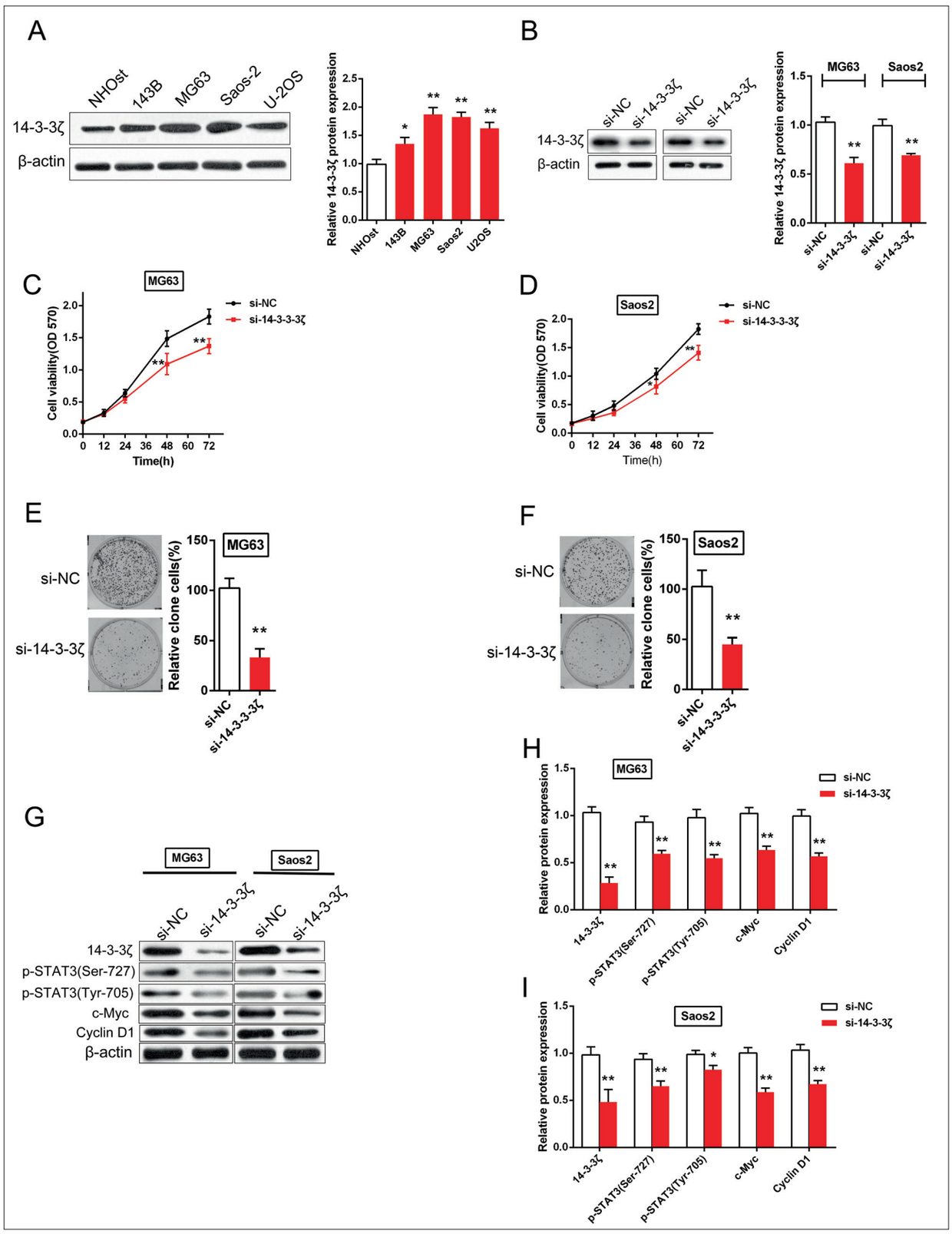

Fig. 1: Expression of 14-3-3ζ in OS cell lines and its promotive effect on OS cell proliferation (A) 14-3-3ζ protein levels in four OS cell lines, 143B, Saos2, MG63 and U2OS, were determined using Western blot assays. (B) si-14-3-3ζ was transfected into MG63 and Saos2 cells to achieve 14-3-3ζ knockdown, as verified using Western blot assays. (C) and (D) si-14-3-3ζ was transfected into MG63 and Saos2 cells; cell viability of MG63 and Saos2 cells was determined using MTT assays. (E) and (F) si-14-3-3ζ was transfected into MG63 and Saos2 cells; cell colony formation capability of MG63 and Saos2 cells was determined using colony formation assays. (G)-(I) si-14-3-3ζ was transfected into MG63 and Saos2 cells; the protein levels of 14-3-3ζ, p-STAT3 (Ser-727 and Tyr-705), c-Myc and Cyclin D1 were determined using Western blot assays. The data are presented as mean±SD of three independent experiments. *P<0.05, **P<0.01.

Previously, STAT3 has been regarded as a key regulator of cell proliferation, differentiation and survival (Catlett-Falcone et al. 1999; Levy and Lee 2002). STAT3 is overexpressed in OS and correlated to poor prognosis in patients with OS (Ryu et al. 2010). Here, we investigated whether factors of STAT3 pathway could be regulated by 14-3-3ζ in OS cell lines. As exhibited by Western blot assays, the protein levels of 14-3-3ζ, p-STAT3 (Ser-727), p-STAT3 (Tyr-705), c-Myc and Cyclin D1 were significantly reduced in si-14-3-3ζ-transfected MG63 and Saos2 cells (Fig.1G-I), indicating 14-3-3ζ regulation of STAT3 pathway-related factors.

2.2. MiR-204 directly binds to the 3'UTR of 14-3-3ζ to inhibit its expression

Next, we investigated the mechanism by which 14-3-3ζ knockdown suppresses OS cell proliferation. As we mentioned, miR-204 has been reported to play a key role in inhibiting proliferation, migration, invasion and epithelial-mesenchymal transition in OS cells (Shi et al. 2015). Here, we investigated whether miR-204 could act on 14-3-3ζ to affect OS cell proliferation. MiR-204 mimics or miR-204 inhibitor was used to achieve ectopic miR-204 expression or miR-204 inhibition, as verified using real-time PCR assays (Fig. 2A). To confirm the interaction between miR-204 and 14-3-3ζ, a wt-*YWHZA* 3'UTR luciferase reporter gene vector and a mut-*YWHZA* 3'UTR luciferase reporter gene vector containing a 5 bp mutation on the predicted binding site of miR-204 in the 3'UTR of *YWHZA* was obtained (Fig. 2B). The indicated vectors were co-transfected with miR-204 mimics or miR-204 inhibitor into HEK293 cells; then the luciferase activity changes were monitored. Results showed that the luciferase activity of wt-*YWHZA* 3'UTR vector was significantly suppressed by miR-204 mimics, amplified by miR-204 inhibitor; after mutation in the predicted binding site of miR-204, the luciferase activity changes were abolished (Fig. 2C), indicating that miR-204 could directly bind to the 3'UTR of *YWHZA*. Further, miR-204 mimics or miR-204 inhibitor was transfected into MG63 and Saos2 cells; the protein levels of 14-3-3ζ were then monitored using Western blot assays. Results showed that ectopic miR-204 expression reduced 14-3-3ζ protein level, whereas miR-204 inhibition increased 14-3-3ζ protein level in both MG63 and Saos2 cells (Fig. 2D and E). These data indicated that miR-204 could negatively regulate 14-3-3ζ through direct binding to the 3'UTR of *YWHZA*.

2.3. MiR-204 inhibits OS cell proliferation through 14-3-3ζ

As demonstrated earlier, 14-3-3ζ knockdown suppressed OS cell proliferation. Then we further confirmed miR-204 regulation of 14-3-3ζ through direct targeting. Here, we evaluated the combined effects of miR-204 and 14-3-3ζ on OS cell proliferation. MG63 and Saos2 cells were co-transfected with miR-204 mimics and pcDNA3.1/14-3-3ζ; then the cell viability and colony formation capability was determined using MTT and colony formation assays. As exhibited by MTT assays, the cell viability was significantly upregulated by 14-3-3ζ, downregulated by miR-204; the suppressive effect of ectopic miR-204 expression on OS cell viability could be partially reversed by 14-3-3ζ (Fig. 3A and B). Further, similar results were obtained from colony formation assays: the colony formation capability of MG63 and Saos2 cell was promoted by 14-3-3ζ, suppressed by miR-204; the suppressive effect of miR-204 on OS cell colony formation capability could be partially reversed by 14-3-3ζ (Fig. 3C and D). These data indicated that miR-204 inhibits OS cell proliferation through 14-3-3ζ.

2.4. STAT3 pathway is involved in miR-204/14-3-3ζ regulation of OS cell proliferation

Since we revealed that miR-204 inhibits OS cell proliferation through 14-3-3ζ, and that 14-3-3ζ knockdown inhibits STAT3 phosphorylation (on Ser-727 and Tyr-705), reduces c-Myc and Cyclin D1 proteins; here we further monitored the protein changes of p-STAT3 (Ser-727 and Tyr-705), c-Myc and Cyclin D1 in response to co-processing miR-204 and 14-3-3ζ in MG63 and Saos2 cells. Results from Western blot assays showed that forced 14-3-3ζ expression significantly increased the protein levels of 14-3-3ζ, p-STAT3 (Ser-727 and Tyr-705), c-Myc and Cyclin D1, ectopic miR-204 expression reduced the protein levels of the indicated factors; moreover, the suppressive effect of miR-204 on these proteins could be partially reversed by 14-3-3ζ. These data further confirmed that miR-204/14-3-3ζ axis act on OS cell proliferation through regulation of STAT3 pathway.

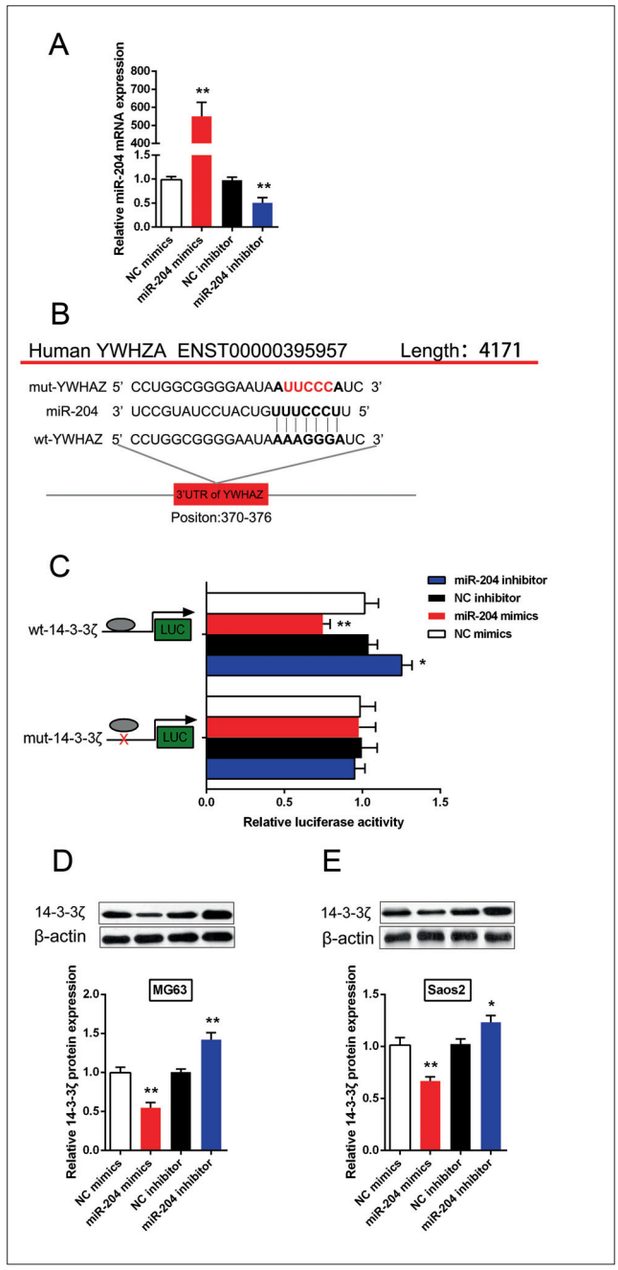

Fig. 2: miR-204 directly binds to the 3'UTR of 14-3-3ζ to inhibit its expression (A) miR-204 mimics and miR-204 inhibitor was used to achieve ectopic miR-204 expression and miR-204 inhibition, as verified using real-time PCR assays. (B) A wt-*YWHZA* 3'UTR luciferase reporter gene vector and a mut-*YWHZA* 3'UTR luciferase reporter gene vector containing a 5 bp mutation on the predicted binding site of miR-204 in the 3'UTR of *YWHZA* was obtained. (C) The indicated vectors were co-transfected with miR-204 mimics or miR-204 inhibitor into HEK293 cells; then the luciferase activity changes were monitored. (D) and (E) miR-204 mimics and miR-204 inhibitor was transfected into MG63 and Saos2 cells; the protein level of 14-3-3ζ in MG63 and Saos2 cells were determined using Western blot assays. The data are presented as mean±SD of three independent experiments. **P*<0.05, ***P*<0.01.

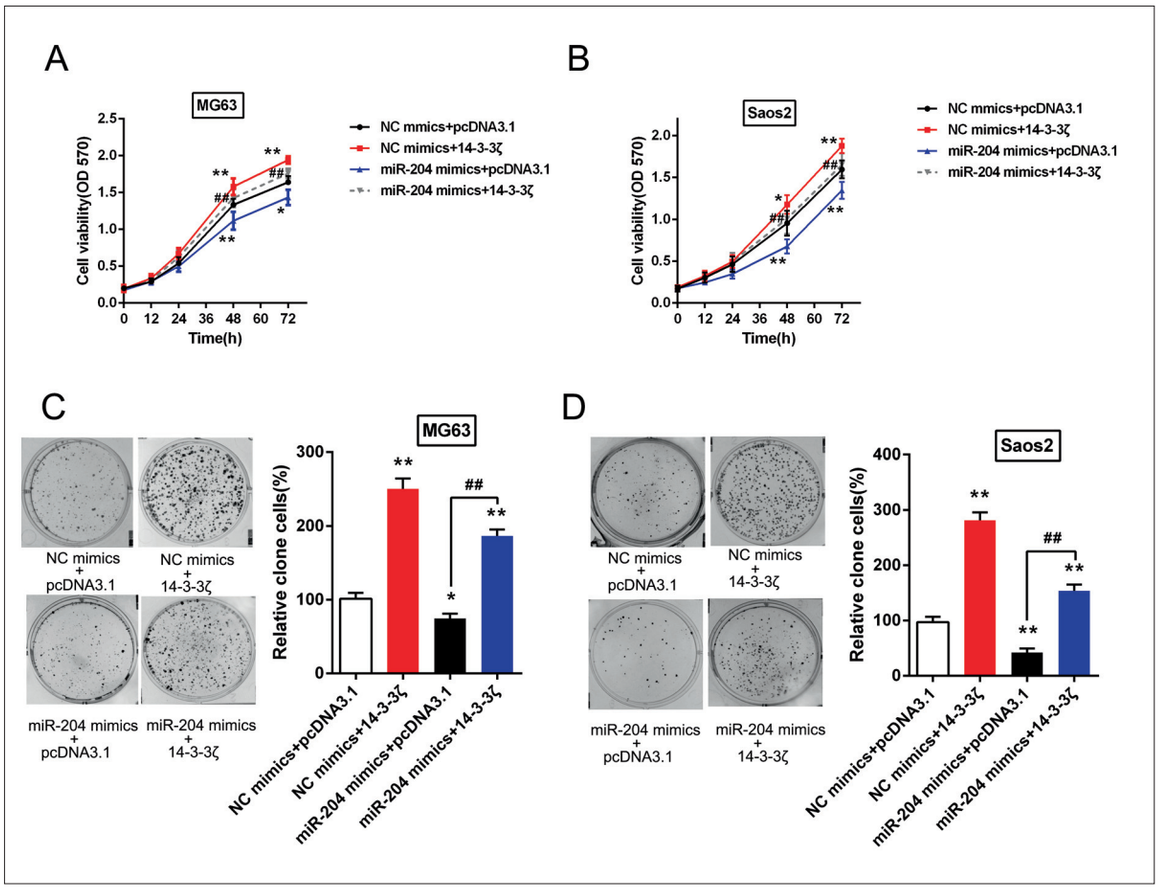

Fig. 3: miR-204 inhibits OS cell proliferation through 14-3-3ζ (A) MG63 and Saos2 cells were co-transfected with miR-204 mimics and pcDNA3.1/14-3-3ζ; the cell viability was determined using MTT assays. (B) MG63 and Saos2 cells were co-transfected with miR-204 mimics and pcDNA3.1/14-3-3ζ; the cell colony formation capability was determined using Colony formation assays. The data are presented as mean±SD of three independent experiments. *P<0.05, **P<0.01, vs NC mimics + pcDNA3.1 group; ##P<0.01, vs miR-204 mimics + pcDNA3.1 group.

3. Discussion

During OS carcinogenesis, a number of factors can promote the tumor cell proliferation. Among these, 14-3-3ζ, a member of the 14-3-3 protein family which acts as a suppressor of apoptosis and has a central role in tumor genesis and progression (Yang et al. 2012), and STAT3, one of the transcription factors reported to play an important role in tumor cell growth, proliferation, apoptosis, and carcinogenesis (Bromberg et al. 1999; Buettner et al. 2002), have been investigated as popular topics. Here we found that the 14-3-3ζ expression on both mRNA and protein levels in four OS cell lines were significantly upregulated, which is consistent with previous studies that the expression of 14-3-3ζ was increased in four NSCLC cell lines compared to that in BEAS-2B (Xue et al. 2016). We further evaluated the functions of 14-3-3ζ in OS cell lines. After 14-3-3ζ knockdown, the cell viability and colony formation capability of OS cells were significantly suppressed, indicating the promotive roles of 14-3-3ζ in regulation of OS cell proliferation.

In previous studies, persistent activation of STAT3 has been implicated in both the induction of cancer and the processes promoting the survival of cancer (Duan et al. 2007; Duan et al. 2006; Lin et al. 2010; Turkson and Jove 2000). Activation of receptor and nonreceptor tyrosine kinases stimulates STAT3 Tyr705 phosphorylation to induce dimerization and increase STAT3 DNA binding activity (Aggarwal et al. 2009; Guschin et al. 1995; Song et al. 2003; Zhong et al. 1994). Phosphorylation of Ser727 is mediated by various serine kinases (e.g., mitogen-activated protein kinases, cyclin-dependent kinases, and protein kinase Cs), and this modification increases STAT3 transcriptional activity by facilitating protein-protein interactions with transcriptional coactivators (Aggarwal et al. 2009; Schuringa et al. 2001; Wen et al. 1995). Activation of STAT3 pathway in OS has been reported. STAT3 and pSTAT3 is overexpressed in osteosarcoma cell lines and tissues (Ryu et al. 2010). Interestingly, 14-3-3ζ has been reported to bind to p-STAT3 (Ser-727), and improve the phosphorylation of STAT3 (Tyr705), to increase its activation (Xue et al. 2016). Since we demonstrated the promotive role of 14-3-3ζ in regulation of OS cell proliferation; here we further investigated whether 14-3-3ζ also promotes STAT3 pathway activation to exert its functions. Consistent with previous studies, 14-3-3ζ knockdown significantly reduced the protein levels of p-STAT3 (both Ser-727 and Tyr-705), indicating the suppressed STAT3 phosphorylation. Moreover, the downstream factors of STAT3 pathway, c-Myc and Cyclin D1, were suppressed by 14-3-3ζ knockdown. These data suggested the involvement of 14-3-3ζ-mediated STAT3 pathway activation during OS cell proliferation.

Given that 14-3-3ζ knockdown suppresses STAT3 pathway activation and OS cell proliferation; targeting 14-3-3ζ to inhibit its expression, thus to inhibit STAT3 pathway and OS cell proliferation should present an efficient way of treating OS. It has been well established that deregulation of miRNAs is tightly associated with the development and progression of various types of human cancer including OS (Ram Kumar et al. 2016). miRNAs play roles in human cancer via mediating the protein expression of their target genes (Yoshitaka et al. 2013). MiR-204, a well-established tumor suppressor, has been reported to inhibit cell migration and invasion of NSCLC cells (Shi et al. 2014) and suppresses tumorigenesis and invasiveness in glioma cells (Ying et al. 2013). More importantly, overexpression of miR-204 significantly inhibited OS cell proliferation, migration and invasion through targeting Sirt1 (Shi et al. 2015). In the present study, we confirmed that

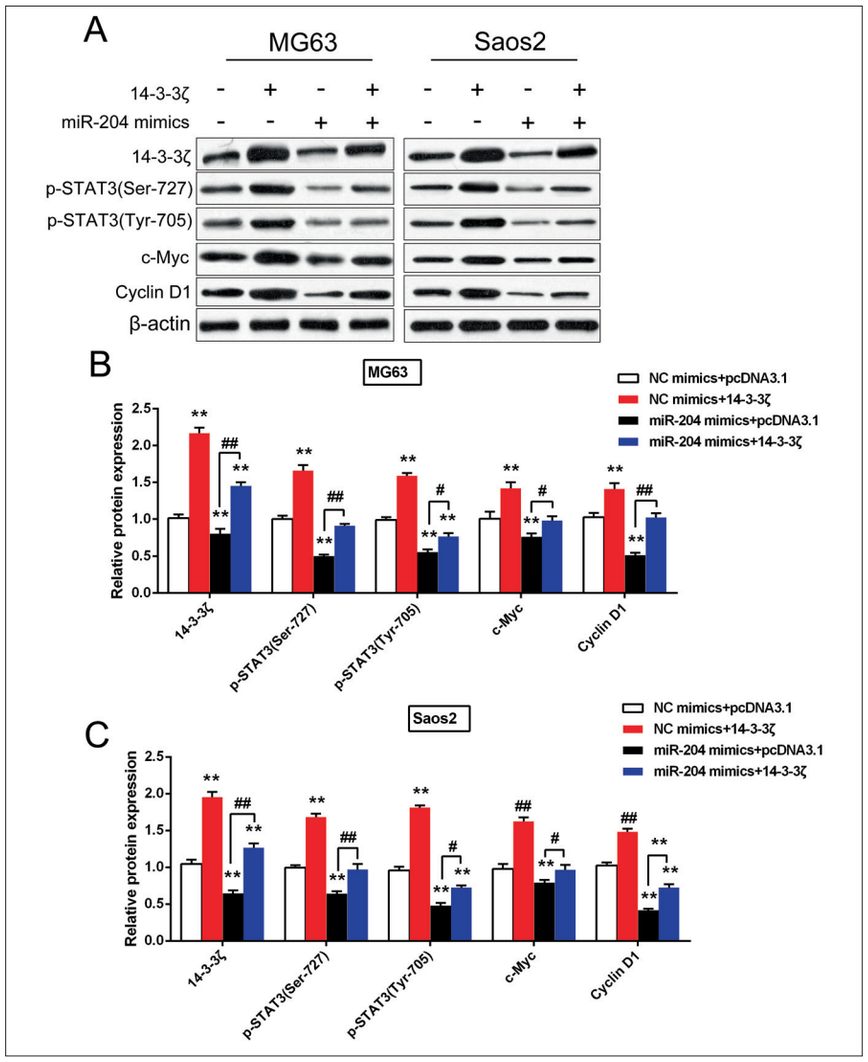

Fig. 4: STAT3 pathway is involved in miR-204/14-3-3ζ regulation of OS cell proliferation (A)-(C) MG63 and Saos2 cells were co-transfected with miR-204 mimics and pcDNA3.1/14-3-3ζ; the protein levels of 14-3-3ζ, p-STAT3 (Ser-727 and Tyr-705), c-Myc and Cyclin D1 were determined using Western blot assays. The data are presented as mean±SD of three independent experiments. $^{**}P<0.01$, vs NC mimics + pcDNA3.1 group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, vs miR-204 mimics + pcDNA3.1 group.

miR-204 could directly bind to the 3'UTR of the *YWHZA* gene, thus to inhibit 14-3-3ζ expression. Further, ectopic miR-204 expression significantly suppressed OS cell proliferation, and this suppressive effect of miR-204 on OS cell proliferation could be partially reversed by 14-3-3ζ overexpression. These data indicated that miR-204 inhibits OS cell proliferation through direct targeting *YWHZA*.

Finally, we also evaluated the functions of miR-204 in activation of STAT3 pathway. Consistent with the proliferation assays, miR-204 overexpression reduced the protein levels of 14-3-3ζ, p-STAT3 (both Ser-727 and Tyr-705), c-Myc and Cyclin D1, and 14-3-3ζ overexpression partially reversed the suppressive effect of miR-204 on the indicated proteins.

Taken together, miR-204 could inhibit OS cell proliferation through direct binding to the 3'UTR of the *YWHZA* gene, thus to inhibit 14-3-3ζ expression and STAT3 pathway activation. In conclusion, our data revealed that the miR-204/14-3-3ζ axis could regulate OS cell proliferation through STAT3 pathway, and implicated miR-204 and 14-3-3ζ as potential therapeutic targets for OS.

4. Experimental

4.1. Cell lines

The human normal osteoblast cell line NHost and four human OS cells lines 143B, Saos2, MG63 and U2OS were obtained from American Type Culture Collection (ATCC, USA). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37 °C and 5% CO_2.

4.2. Cell transfection

Cells were routinely maintained in a 37 °C humidified atmosphere of 5% CO_2. si-14-3-3ζ, pcDNA3.1/14-3-3ζ, miR-204 mimics or miR-204 inhibitor (Genepharma, Shanghai, China) were transfected or co-transfected into MG63 and Saos2 cells using Lipofectamine2000 (Invitrogen, CA, USA). Cells were plated in 96-well plates and transfected for 24 h or 48 h, and then used for further assays.

4.3. RNA extraction and SYBR green quantitative PCR analysis

Total RNA was extracted by using Trizol reagent (Invitrogen, CA, USA). miRNA expressions in cells were detected using a Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China). U6 expression was used as endogenous control. SYBR green qPCR assay (Takara, Dalian, China) was used to measure the 14-3-3ζ expression. The relative fold changes of candidate genes were analyzed using the $2^{-\Delta\Delta CT}$ method.

4.4. Western blot analysis

Cells were lysed in RIPA buffer with 1% PMSF. SDS-PAGE was conducted to separate the cellular proteins. Then the proteins were transferred onto PVDF membrane. The following antibodies were used to probe with the membranes at 4 °C overnight: 14-3-3ζ (rabbit polyclonal, ab51129, Abcam, Cambridge, MA, USA), p-STAT3 (Ser-727, rabbit monoclonal, Cat# E121-31, Abcam), p-STAT3 (Tyr-705, rabbit monoclonal, Cat# EP2147Y, Abcam), c-Myc (rabbit monoclonal, Cat# Y69, Abcam), Cyclin D1 (rabbit monoclonal, Cat# EPR2241, Abcam) and β-actin (mouse monoclonal, Cat# mAbcam 8226, Abcam). Signals were visualized using ECL Substrates and their digital readings are shown in related figures (Millipore, MA, USA). β-actin was used as endogenous control.

4.5. MTT assay

Cell viability was evaluated using a modified MTT assay. Transfected or non-treated cells were assessed at five time points (on day 1, 2, 3, 4 and 5) after seeding into

96-well culture plates (2×10^3 cells/well). Briefly, quantification of mitochondrial dehydrogenase activity was achieved by the enzymatic conversion of MTT [3-(4, 5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, MO, USA] to a colored formazan product. MTT (10 µl, 10 mg/ml) was added to the cells, incubated for 4 h, and the reaction was terminated by removal of the supernatant and addition of 100 µl DMSO to dissolve the formazan product. After 0.5 h, the optical density (OD) of each well was measured at 570 nm using a plate reader (ELx808 Bio-Tek Instruments, City, ST, USA).

4.6. Colony formation assay

Transfected or non-treated cells were seeded and suspended in RPMI-1640 containing 0.35% low-melting agarose and plated onto 0.6% agarose in six-well culture plates at a density of 1×10^5 cells per dish. The plates were incubated for two weeks at 37 °C in a 5% CO_2 incubator, and the number of colonies was counted after staining with 0.1% crystal violet solution. Colonies with more than 50 cells were manually counted.

4.7. Luciferase activity

YWHZA is the gene name of 14-3-3ζ. A wt-*YWHZA* 3'UTR luciferase reporter gene vector and a mut-*YWHZA* 3'UTR luciferase reporter gene vector containing a 5 bp mutation on the predicted binding site of miR-204 in the 3'UTR of *YWHZA* was obtained from Yrbio Co, ltd (Changsha, China). HEK293 cells (ATCC, USA) were cultured overnight after being seeded into a 24-well plate, co-transfected with the indicated vectors and miR-204 mimics or miR-204 inhibitor. 48 h after transfection, a Dual Luciferase Reporter Assay System (Promega, USA) was used to determine the luciferase activity.

4.8. Statistical analysis

All data from three independent experiments were expressed as mean±SD and processed using SPSS 17.0 statistical software. Comparisons between two groups were conducted using two-tail Student's T-test. For multi-group comparisons, statistical significance was determined using one-way ANOVA followed by a post hoc Tukey's test. Differences were considered to be statistically significant when P value is less than 0.05.

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Conflicts of interest: The authors declare that there is no conflict of interest.

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