■ ONCOLOGY

Silencing of translation initiation factor eIF3b promotes apoptosis in osteosarcoma cells

Y. J. Choi,
Y. S. Lee,
H. W. Lee,
D. M. Shim,
S. W. Seo

Sungkyunkwan University, Seoul, Korea

Objectives

Eukaryotic translation initiation factor 3 (eIF3) is a multi-subunit complex that plays a critical role in translation initiation. Expression levels of eIF3 subunits are elevated or decreased in various cancers, suggesting a role for eIF3 in tumorigenesis. Recent studies have shown that the expression of the eIF3b subunit is elevated in bladder and prostate cancer, and eIF3b silencing inhibited glioblastoma growth and induced cellular apoptosis. In this study, we investigated the role of eIF3b in the survival of osteosarcoma cells.

Methods

To investigate the effect of eIF3b on cell viability and apoptosis in osteosarcoma cells, we first examined the silencing effect of eIF3b in U2OS cells. Cell viability and apoptosis were examined by the Cell Counting Kit-8 (CCK-8) assay and Western blot, respectively. We also performed gene profiling to identify genes affected by eIF3b silencing. Finally, the effect of eIF3b on cell viability and apoptosis was confirmed in multiple osteosarcoma cell lines.

Results

eIF3b silencing decreased cell viability and induced apoptosis in U2OS cells, and by using gene profiling we discovered that eIF3b silencing also resulted in the upregulation of tumour necrosis factor receptor superfamily member 21 (TNFRSF21). We found that TNFRSF21 over-expression induced cell death in U2OS cells, and we confirmed that eIF3b silencing completely suppressed cell growth in multiple osteosarcoma cell lines. However, eIF3b silencing failed to suppress cell growth completely in normal fibroblast cells.

Conclusion

Our data led us to conclude that eIF3b may be required for osteosarcoma cell proliferation by regulating TNFRSF21 expression.

■ Y. J. Choi, PhD, Senior Researcher,
■ Y. S. Lee, MSc, Researcher,
■ D. M. Shim, BSc, Researcher,
■ S. W. Seo, MD, PhD, Professor, Department of Orthopaedic Surgery, Samsung Medical Center, Sungkyunkwan University, 50, Ilwon-dong, Gangnam-gu, 135-710, Seoul, South Korea.
■ H. W. Lee, MSc, Researcher, Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, 50, Ilwon-dong, Gangnam-gu, 135-710, Seoul, South Korea.

Correspondence should be sent to S. W. Seo; email: sungwseo@gmail.com

Article focus

■ Could eIF3b silencing mediate apoptosis in osteosarcoma?
■ What is the key downstream effector which mediates apoptosis in eIF3b-silenced osteosarcoma?

Key messages

■ Silencing of eIF3b increases TNFRSF21 expression, and TNFRSF21 induces cell death.

Strengths and limitations

■ All of these results were obtained by *in vitro* study.

Introduction

The control of protein translation is important for cancer development and progression. Global, as well as specific, mRNA translational regulation can promote tumour cell survival, angiogenesis, transformation, invasion and metastasis.[1] Translation initiation is a rate-limiting step in protein synthesis from mRNA. A multitude of eukaryotic translation initiation factors (eIFs) are involved in the regulation of translation initiation.[2] Several eIFs are overexpressed in cancer cells, and a considerable amount of research has been done to inhibit their function. For example, several human cancers exhibit eIF4E,

eIF4A, and eIF5A overexpression, and these proteins have been considered targets of anticancer therapies.[3-6] The function of eIF4E has been inhibited by using specific antisense oligonucleotides (4E-ASO)[7] and ribavirin, a nucleoside inhibitor.[8] Moreover, pateamine A,[9] silvestrol,[10] and hippuristanol[11] have been reported to target eIF4A. In the case of eIF5A, N1-guanyl-1,7-diaminoheptane[12] and ciclopirox olamine[13] inhibited eIF5A by preventing its hypusination, a post-translational modification that is required for the full activity of eIF5A.

Another eIF called eIF3 is a multi-subunit complex, which plays a central role in translation initiation. The eIF3 complex is composed of at least 13 subunits,[14,15] and the eIF3a,[16-20] eIF3c[21] and eIF3h[22] subunits have been shown to be overexpressed in some carcinomas and in testicular seminoma. On the other hand, the eIF3f and eIF3e subunits have been reported to have decreased expression in melanoma and carcinoma cells.[23,24] Indeed, eIF3 subunits can regulate the expression of proteins involved in cell cycle control. The eIF3a subunit negatively regulates the expression of p27, a cyclin dependent kinase (CDK) inhibitor.[25] In addition, the expression of the proto-oncogene Myc correlated with the levels of eIF3h.[22,26]

The eIF3b subunit has been shown to be overexpressed in cancers of the prostate and bladder, and its overexpression has been linked to cancer prognosis.[27] Although siRNA-mediated silencing of eIF3b inhibited the proliferation of colon cancer cells and glioblastoma,[28,29] the potential role of eIF3b in the aggressive bone cancer osteosarcoma has not been elucidated. In this study, we investigated the effect of inhibiting eIF3b function on the survival of several osteosarcoma cell lines.

Materials and Methods

Cell culture. Three human osteosarcoma cell lines were used for the experiments and those were purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). Human osteosarcoma cell lines (U2Os, MG63 and 143B) were authenticated using Short Tandem Repeat (STR) analysis. When compared with the reference profile by ATCC, cell lines with ≥ 80% match were considered to be related (Supplementary Table i). U2OS cells were grown in Modified Eagle's medium (MEM). MG63 and 143B cells were grown in Dulbecco's Modified Eagle's medium (DMEM). As a control, the human foetal fibroblast cell, HDF-f (ScienCell Research Laboratories, Carlsbad, California) was used. Normal fibroblasts were grown in DMEM. All media were supplemented with 10% foetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, Utah) and 1% antibiotic/antimycotic solution (Gibco Laboratories, Gaithersburg, Maryland). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.
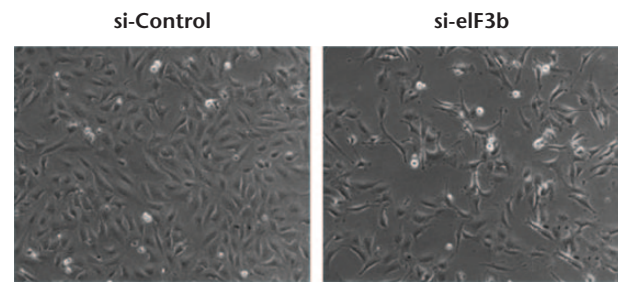
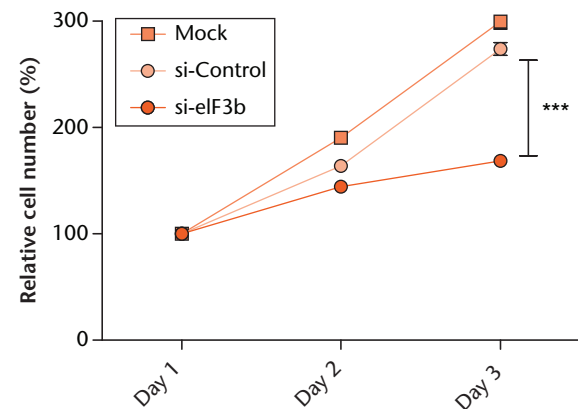**si-Control**   **si-eIF3b**
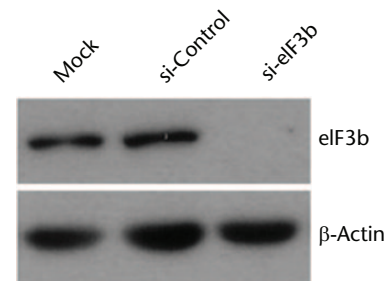

Fig. 1a

Fig. 1b

Fig. 1c

Knockdown of eIF3b inhibits osteosarcoma cell growth. U2OS cells were transfected with control or eIF3b siRNA. a) Cell density of U2OS cells, three days after transfection (× 100). b) Cell viability was measured for three days. Asterisks indicate *** $p < 0.001$ in two tailed t-tests (comparison between control and eIF3b siRNA at day three). Analyses were performed in triplicate. c) eIF3b silencing was confirmed by Western blot analysis. βActin was used as an internal control.

eIF3b siRNA transfection. The eIF3b siRNA sequence was sense: (5'-GGAGAGAAAUUCAAGCAAA(dTdT)-3') and antisense: (5'-UUUGCUUGAAUUUCUCUCC(dTdT)-3'). The siRNA for eIF3b was designed based on the reported target site.[28] Negative control siRNA with scrambled sequence (SN1003) and the eIF3b siRNA were purchased from Bioneer Corp. (Daejeon, Korea). The control and eIF3b siRNAs were transfected using Lipofectamine RNAiMAX Reagent (Life Technologies, Carlsbad, California) according to the manufacturer's instructions.
Plasmid transfection. The tumor necrosis factor receptor superfamily member 21 (TNFRSF21)-expressing plasmid, pcDNA3-TNFRSF21-Flag (HG10175-CF), was purchased
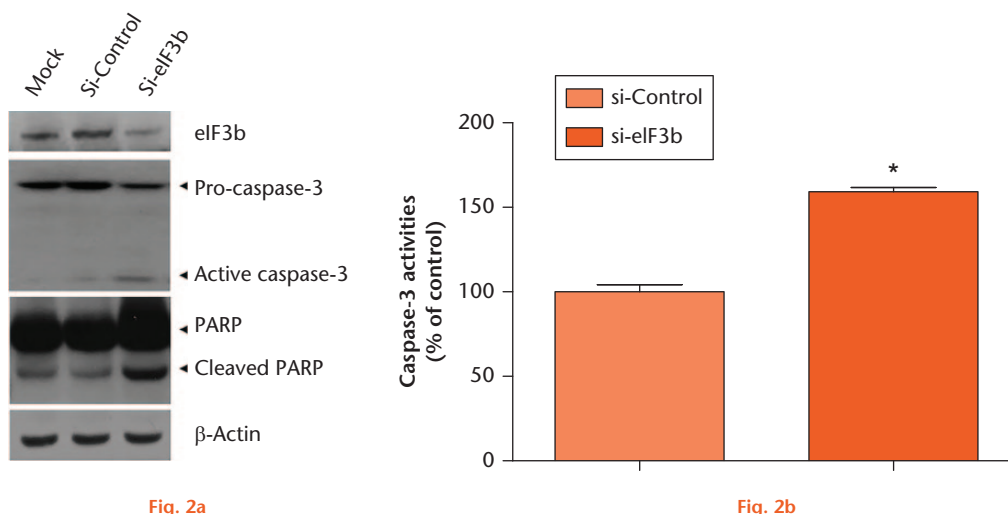
Fig. 2a **Fig. 2b**

Knockdown of eIF3b induces apoptosis in U2OS cells. a) Three days after transfection of eIF3b siRNA, the level of active caspase-3 and cleaved poly ADP ribose polymerase (PARP) fragment was examined by Western blot analysis. Increased levels of cleaved caspase-3 and PARP fragment were detected in si-eIF3b-transfected U2OS cells. b) Caspase-3 activity was measured three days after siRNA transfection (* $p < 0.05$ in two tailed t-tests).

from Sino Biological Inc. (Beijing, China). The sequence of the plasmid was confirmed by Sanger sequencing. Plasmids were transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

Cell viability assay. Cell viability was monitored by Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). For each assay, 1×10^3 cells were plated onto 96-well plates 24 hours after siRNA or expression plasmid transfection and were grown for the indicated days. CCK-8 solution (10 µl) was added to each well and incubated for one hour. The absorbance at 450 nm was measured using a SpectraMax Plus 384 instrument (Molecular Devices, München, Germany).

Western blot. Cells were washed with ice-cold phosphate buffered saline (Biosesang, Gyeonggi, Korea) and the cell lysate was prepared in lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% NP-40). Protein concentrations were determined by Bradford protein assay reagent (Bio-Rad, Hercules, California). Equal amounts of protein were subjected to SDS-PAGE followed by transferral to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was incubated with 5% skimmed milk in TBST (Biosesang, Gyeonggi, Korea) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for one hour at room temperature. After being washed with TBST, the membrane was incubated overnight at 4°C with 5% bovine serum albumin in TBST containing the appropriate primary antibodies. After being washed with TBST, the membrane was then incubated with 5% skimmed milk in TBST containing the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) for one hour at room temperature. After being washed with TBST, the membrane was soaked in Pierce ECL Western Blotting Substrate (Thermo

Fisher Scientific Inc., Rockford, Illinois) and exposed to radiograph film. The following antibodies were used: Anti-β-actin (Sigma-Aldrich, St. Louis, Missouri), anti-eIF3b (Abcam, Cambridge, United Kingdom), anti-poly ADP ribose polymerase (PARP), anti-caspase-3, anti-rabbit IgG-HRP, anti-mouse IgG-HRP (Cell Signaling Technology Inc., Danvers, Massachusetts).

Microarray analysis. Gene expression profiling was performed with GeneChip Human Gene 2.0 ST Arrays (Affymetrix, Santa Clara, California). Total RNA was extracted using RNeasy (Qiagen Inc., Valencia, California) and RNA purity and integrity were evaluated by the OD 260/280 ratio and analysed by Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, California). The array process was executed by Macrogen Inc. (Seoul, Korea). cDNA was synthesised using the GeneChip WT (Whole Transcript) Amplification Kit as described by the manufacturer. The sense cDNA was then fragmented and biotin-labelled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip WT Terminal Labeling Kit. Approximately 5.5 µg of labelled DNA target was hybridised to the Affymetrix GeneChip Human 2.0 ST Array at 45°C for 16 hours. Hybridised arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (Affymetrix). The experiment was performed in three replicates. The raw data were extracted automatically in Affymetrix data extraction protocol using the software provided by Affymetrix GeneChip Command Console Software (AGCC, Affymetrix). After importing CEL files, the data were summarised and normalised with the robust multi-average (RMA) method implemented in the Affymetrix Expression Console Software (Affymetrix). The comparative analysis was carried out using an local pooled error (LPE) test and fold change in which the null hypothesis was that no difference existed between the
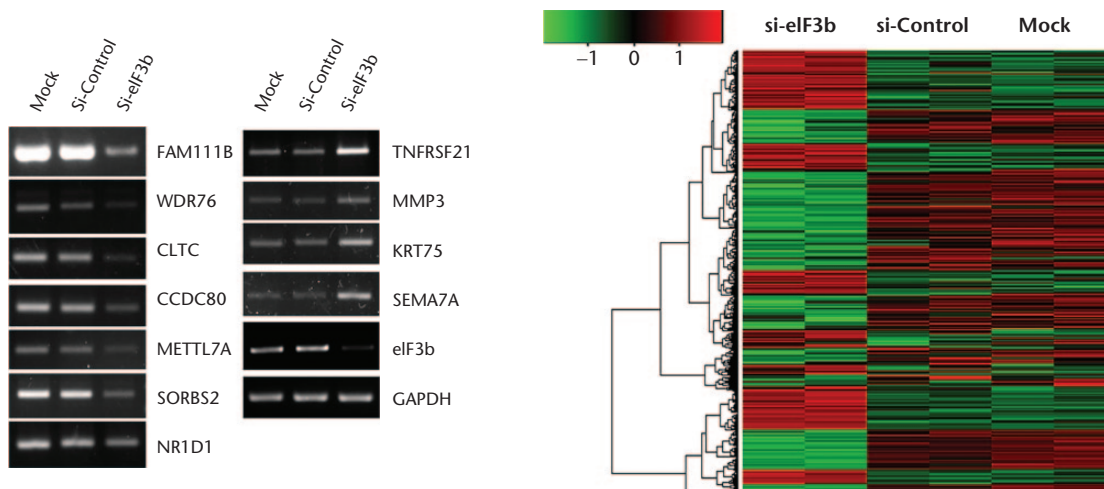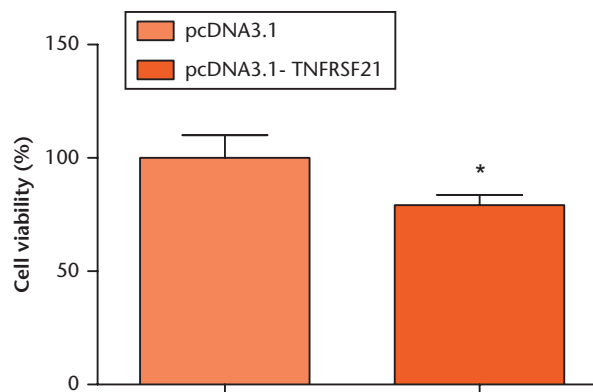
Fig. 3a

Fig. 3b

Fig. 3c

Gene expression profiling in U2OS cells in response to knockdown of eIF3b. a) Two days after transfection of eIF3b siRNA, RNA was isolated and subjected to microarray analysis (duplicates). b) The heat map of eIF3b siRNA-transfected U2OS cells demonstrated significantly up- and downregulated genes compared with control siRNA-transfected or untransfected cells. To validate gene expression profiles from the microarrays, semi-quantitative reverse transcription polymerase chain reaction was performed against the genes listed in Supplementary Table iv. c) U2OS cells were transfected with pcDNA3.1 or pcDNA3-TNRSF21. Three days after transfection, cell viability was measured. (* p < 0.05 in two-tailed *t*-test).

two groups. The false discovery rate (FDR) was controlled by adjusting the p-value using the Benjamini-Hochberg algorithm. All statistical tests and visualisation of differentially expressed genes were conducted using R statistical language v.3.0.2 (R foundation, Vienna, Austria).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted using RNeasy (Qiagen), and 500 ng of RNA was reverse-transcribed with SuperScript III (Life Technologies) according to the manufacturer's instructions. PCR was performed with 1 µl of cDNA using AccuPower HotStart PCR PreMix (Bioneer Corp.). The PCR conditions were 95°C for five minutes, then 30 or 33 cycles (95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds), and finally 72°C for seven minutes. Primer sequences used for RT-PCR are provided in Supplementary Tables ii and iii.

Caspase-3 activity analysis. U2OS cells were transfected with control or eIF3b siRNA. Three days after transfection, cells were harvested for caspase-3 activity analysis using

a Caspase-3 Colorimetric Activity Assay Kit (Chemicon International Inc., Temecula, California). Caspase-3 activity was measured using 30 µg of lysate by detecting the chromophore p-nitroaniline, which was cleaved from the labelled substrate N-acetyl-Asp-Glu-Val-Asp p-nitroanilide.

Statistical analysis. Data are expressed as mean and standard deviation (SD) from three independent experiments. Analyses were performed using the GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California). The significance of the statistical comparisons was determined via two-tailed t-tests. A p-value < 0.05 were determined to be statistically significant and marked by asterisks (* p < 0.05; ** p < 0.01; *** p < 0.001; ns, not significant).

Results

eIF3b silencing reduces osteosarcoma cell growth. To determine whether eIF3b is important for osteosarcoma, U2OS cells were transfected with siRNA-targeting eIF3b

to decrease its levels, and cell viability assays were performed. Indeed, the growth of the cells was significantly inhibited when eIF3b was knocked down by siRNA (Figs 1a and 1b). We confirmed the depletion of eIF3b by siRNA using Western blot analysis (Fig. 1c). These results indicate that eIF3b is required for robust osteosarcoma cell growth.

eIF3b silencing activates apoptosis pathway in U2OS cells. To examine whether eIF3b silencing induces not only cell growth inhibition but also apoptosis, we analysed activation of apoptosis related proteins. Western blot analysis showed that eIF3b silencing increased PARP and caspase-3 cleavage (Fig. 2a), a readout of an active apoptotic cellular programme. Moreover, we also found
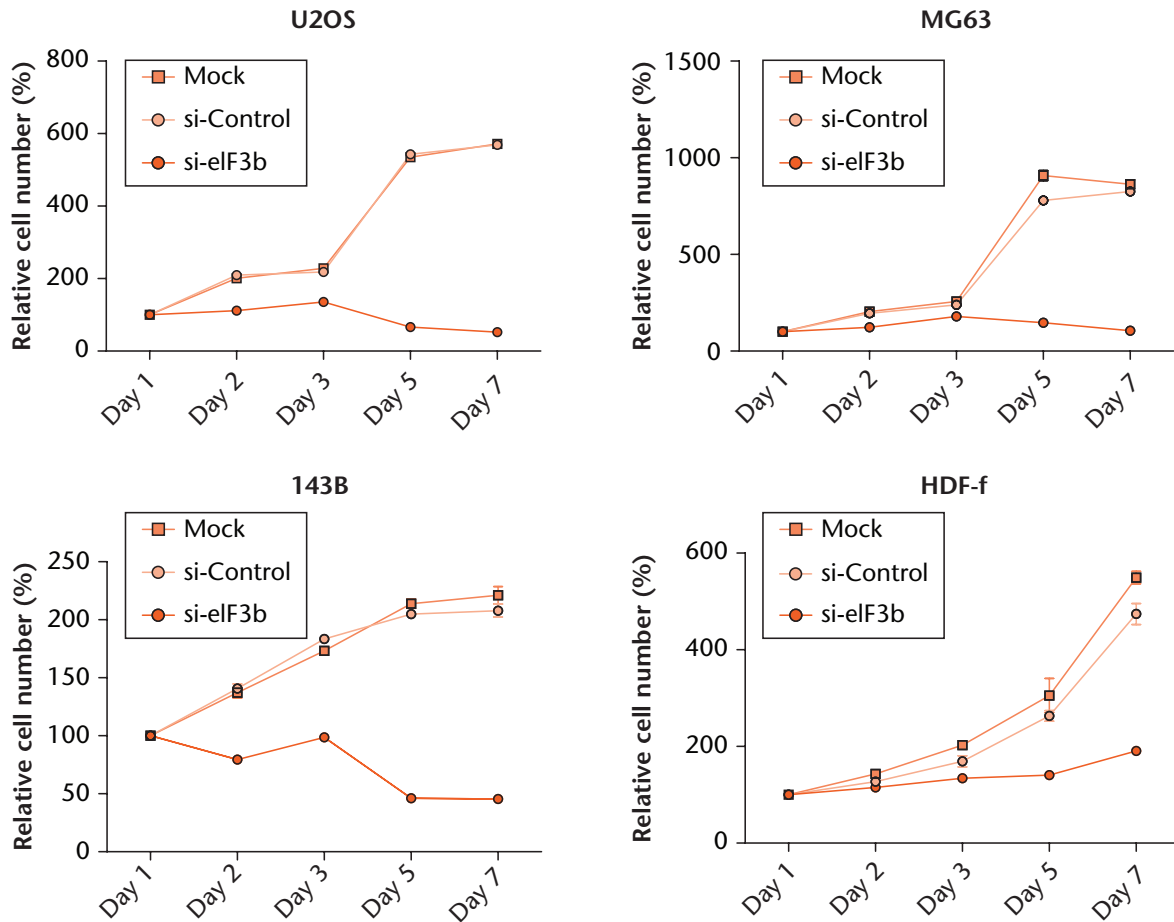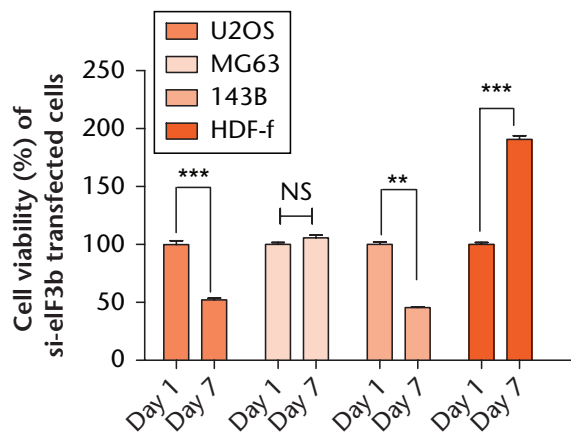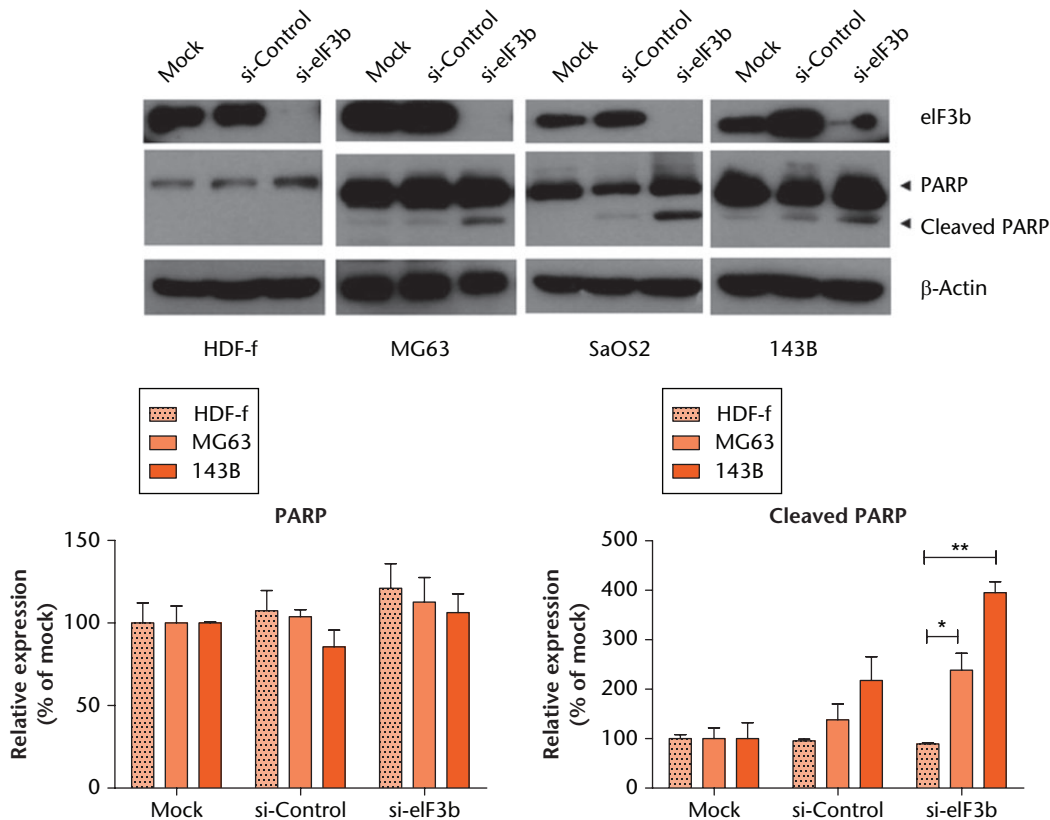

Fig. 4a

Fig. 4b

Fig. 4c

TNFRSF21 induces cell death in U2OS cells. a) Cells were transfected with control or eIF3b siRNA. After transfection, cell viability was measured for seven days. Day one indicates 24 hours after transfection. b) Cell viability at day one and seven after eIF3b siRNA transfection was compared. c) Three days after transfection, the level of cleaved poly ADP ribose polymerase fragment was examined by Western blot analysis (top) and Western blot band was quantified (bottom). The significance of the statistical comparisons was determined via two-tailed t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant).

that caspase-3 activity was increased in cells depleted of eIF3b (Fig. 2b).

TNFRSF21 upregulation plays a role in the apoptosis induced by eIF3b silencing. We performed microarray analyses to screen for genes that are regulated by eIF3b. The microarray heat map showed different gene expression profiles between the eIF3b-silenced samples and the control non-silenced samples (i.e. si-Control and Mock) (Fig. 3a). We detected 542 genes that were upregulated, as well as 656 downregulated genes, when eIF3b was depleted from cells using siRNA. Furthermore, 101 genes displayed expression levels that were affected more than twofold by eIF3b silencing ($p < 0.05$). Eleven genes that showed the largest differences in expression levels were confirmed by semi-quantitative RT-PCR (Fig. 3b). Among these genes, we chose to examine TNFRSF21 as a potential regulator for the apoptosis induced by eIF3b silencing because TNFRSF21 is directly involved in cell death by inducing caspase-3 activity. We therefore performed cell viability assays to examine whether TNFRSF21 could induce cell death in U2OS cells and indeed, overexpression of TNFRSF21 did induce cell death in U2OS cells (Fig. 3c).

However, when the apoptotic effect of TNFRSF21 overexpression was compared with that of eIF3b silencing, TNFRSF21 was not as strong an inducer of apoptosis compared with cells depleted of eIF3b. Therefore, although TNFRSF21 is a downstream effector of eIF3b silencing, additional effectors must participate in the apoptosis induced by eIF3b siRNA.

Effect of eIF3b silencing in multiple osteosarcoma cell lines. To further confirm the effect of eIF3b silencing on the proliferation of osteosarcoma cells, we performed proliferation assays using the U2OS, MG63 and 143B cell lines. Strikingly, eIF3b silencing completely inhibited cell growth in each osteosarcoma cell line. Normal fibroblast cells (HDF-f) also exhibited growth inhibition due to eIF3b silencing (Fig. 4a). However, the growth of HDF-f was recovered at a later time point, by day 7 (Fig. 4b). We analysed PARP cleavage to evaluate the activity of apoptosis pathway induced by eIF3b silencing in those cell lines. PARP expression was significantly higher in osteosarcoma cell lines when compared with the HDF-f cell lines. cCleaved PARP was observed to be significantly higher among eIF3b siRNA-treated osteosarcoma cell lines, but not in HDF-f cells (Fig. 4c).

Discussion

Several studies have shown that silencing of eIF3b inhibited the proliferation of different types of human cancers including bladder, prostate, and colon, as well as glioblastoma.[27-29] However, whether eIF3b has a role in osteosarcoma proliferation has not previously been studied. In this study, we investigated the effect of altering eIF3b levels on osteosarcoma cell fate. eIF3b silencing inhibited U2OS cell proliferation while overexpression of eIF3b enhanced cell proliferation.

eIF3b is involved in protein synthesis, and eIF3b depletion can globally inhibit protein synthesis.[27] Given that cell proliferation requires protein synthesis, the inhibited cell proliferation by eIF3b silencing might be a consequence of protein synthesis inhibition. However, in this study we observed activation of the caspase-3/PARP pathway by eIF3b silencing in U2OS cells. Moreover, other osteosarcoma cell lines, including MG63 and 143B, also showed complete growth inhibition and cell death by eIF3b silencing. In normal fibroblast cells (HDF-f), the growth inhibition was not complete and PARP activation was not detected. However, further study is needed toconfirm that eIF3b silencing is non-toxic for normal cells.

By using gene expression profiling, we found that the expression of TNFRSF21 was increased by eIF3b silencing. TNFRSF21 possesses a death domain and is known as Death Receptor 6 (DR6). It has been reported that TNFRSF21 overexpression induces apoptosis in a variety of cell types,[30-33] and promotes apoptosis through activation of caspase-3.[31,33] The apoptotic effect of TNFRSF21 has been shown in M17 neuroblastoma cells, human H4 neuroglioma cells, human cervical cancer HeLa cells and human embryonic kidney 293 (HEK293) cells.[30-32] However, apoptosis was not induced by TNFRSF21 overexpression in human breast cancer MCF7 cells.[32] We confirmed that cell death is induced by TNFRSF21 in U2OS cells, likely in part by the activation of caspase-3 in response to eIF3b silencing. However, TNFRSF21 is unlikely to be the main effector that induces cell death by eIF3b silencing. The rate of cell death induced by TNFRSF21 was less than that induced by eIF3b silencing. Thus, there may be other effectors that induce apoptosis when eIF3b expression is silenced. Gene profiling also revealed several genes that were up- or downregulated by eIF3b silencing. Even though we could not identify the relationship between these genes and cell death, the gene expression changes will likely give clues for further studies of eIF3b function in osteosarcoma.

The strength of our study is that we first showed the cross-talk between TNFRSF21 and eIF3b. Moreover, even though our results are somewhat limited due to *in vitro* approaches, targeting eIF3b in osteosarcoma may be beneficial because normal fibroblast cell death was not induced by eIF3b silencing. Further study using animal model will be required to confirm the eIF3b targeting effect.

Supplementary material

ë   A figure showing overexpression of eIF3b enhanced survival into U2OS cells and tables showing cell line authentication result, sequences and primers which have used for the studies and a list of genes used are available alongside this article at www.bjr.boneandjoint.org.uk

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