Suppression of antitumor cytokine IL-24 by PRG4 and PAI-1 may promote myxoid liposarcoma cell survival

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Abstract. Suppression of the antitumor cytokine interleukin-24 (IL-24) is critical for the survival of myxoid liposarcoma (MLS) cells. It has been previously demonstrated by the authors that an MLS-specific chimeric oncoprotein, translocated in liposarcoma-CCAAT/enhancer-binding protein homologous protein (TLS-CHOP), supresses *IL24* mRNA expression via induction of proteoglycan 4 (PRG4) to sustain MLS cell proliferation. However, IL-24 has also been revealed to be suppressed by the ubiquitin-proteasome system in human ovarian and lung cancer cells. Therefore, the aim of the present study was to elucidate the mechanism of IL-24 suppression in MLS cells. The results revealed that the proteasome inhibitor, MG-132, induced cell death in MLS cells *in vitro*; this effect was reduced following IL-24 knockdown. This indicated that proteasomal degradation of IL-24 may be an important process for MLS cell survival. In addition, it was also previously revealed by the authors that knockdown of plasminogen activator inhibitor-1 (PAI-1), a TLS-CHOP downstream molecule, suppressed the growth of MLS cells, thus instigating the investigation of the effect of PAI-1 on IL-24 expression in MLS cells. Double knockdown of PAI-1 and IL-24 negated the growth-suppressive effect of PAI-1 single knockdown in MLS cells. Interestingly, PAI-1 single knockdown did not increase the mRNA expression of *IL24*, but it did increase the protein abundance of IL-24, indicating that PAI-1 suppressed IL-24 expression by promoting its proteasomal degradation. Moreover, treatment of MLS cells with a PAI-1 inhibitor, TM5275, induced IL-24 protein expression and apoptosis. Collectively, the results of the present as well as previous studies indicated that IL-24 expression may be suppressed at the transcriptional level by PRG4 and at the protein level by PAI-1 in MLS cells. Accordingly, PAI-1 may represent an effective therapeutic target for MLS treatment.

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Introduction

Myxoid liposarcoma (MLS) is the second most common type of liposarcoma, accounting for ~10% of all adult soft tissue sarcomas. MLS typically develops in the deep soft tissue of the extremities and tends to metastasize to nonpulmonary soft tissue sites (1,2). Surgical resection with or without radiotherapy is the standard treatment modality for localized MLS. Although most MLS patients have favorable prognoses, a subgroup of patients develops local recurrence and metastasis (2-4). Most cases of MLS are characterized by specific chromosomal translocations that generate the chimeric oncogenes encoding translocated in liposarcoma (TLS)-CCAAT/enhancer-binding protein homologous protein (CHOP), while cases with translocations in Ewing's sarcoma (EWS)-CHOP are rare (5-7). TLS is also called fused in sarcoma (FUS), and CHOP is also known as DNA-damage-inducible transcript 3 (DDIT3) or growth arrest and DNA damage-inducible gene 153 (GADD153). These chimeric oncoproteins function as MLS-specific transcription factors that inhibit adipocytic differentiation and promote malignant transformation and tumor progression (8-12).

The expression of the antitumor cytokine interleukin-24 (IL-24) is progressively decreased during the development of melanoma from melanocytes, suggesting its tumor-suppressive function in melanoma progression (13-16). Indeed, ectopic expression of IL-24 has been revealed to induce growth arrest and apoptotic cell death in human malignant tumor cells from diverse origins (17-19); however, it has minimal lethal effects on normal cells (20). Suppression of IL-24 has also been demonstrated to be critical for MLS cell survival. It was previously reported by the authors that proteoglycan 4 (PRG4), a downstream molecule of TLS-CHOP and EWS-CHOP, supressed *IL24* mRNA expression to sustain MLS cell proliferation (9,19,21). In addition, the abundance of IL-24 protein was revealed to be decreased by the ubiquitin-proteasome system in human ovarian and lung cancer cells (22). However, it remains unknown whether

proteasomal degradation of IL-24 is required for MLS cell survival.

Plasminogen activator inhibitor-1 (PAI-1) has been revealed to be highly expressed in various types of cancer and to promote tumor growth and metastasis as well as angiogenesis (23). It has been previously demonstrated that TLS-CHOP induced the expression of PAI-1, whereas PAI-1 knockdown suppressed MLS cell proliferation (24). However, the molecular mechanism underlying the growth-suppressive effects of PAI-1 knockdown remains unclear.

The present study investigated whether IL-24 was regulated by the ubiquitin-proteasome system in MLS cells and whether PAI-1 was associated with the underlying mechanism. The effects of a pharmacological inhibitor of PAI-1 on MLS cells was also evaluated.

Materials and methods

Cell culture. The MLS-derived cell lines, 2645/94 and 1955/91, were kindly provided by Professor David Ron (University of Cambridge, Cambridge, UK) and cultured in Dulbecco's modified Eagle's medium (product no. D5796; Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Biowest) at 37˚C in a humidified incubator with 5% CO_2. The cell lines were tested for mycoplasma contamination using Cycleave PCR Mycoplasma Detection Kit (Takara Bio, Inc.). Phase-contrast images were captured using an inverted microscope (ECLIPSE TS100; Nikon Corporation) and a Microscope Camera Control Unit (DS-L3; Nikon Corporation). For cell quantitation, cells were harvested from the wells of a 12-well plate and resuspended in phosphate-buffered saline (PBS). An equal volume of Trypan blue solution (0.4%; Sigma-Aldrich Corporation; Merck KGaA) was then added to each sample at room temperature, and immediately viable cell numbers were calculated from four independent counts using a hemocytometer.

Small interfering RNA (siRNA) transfection. 2645/94 and 1955/91 cells grown to ~10% confluence were transfected with 10 nM of siRNAs using the Lipofectamine® RNAiMAX Transfection Reagent and Opti-MEM I Reduced Serum Medium (both from Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The cells were then cultured at 37˚C in a humidified incubator with 5% CO_2 until the cells were harvested for cell quantitation or preparation of protein or RNA samples. The target nucleotide sequences of *PRG4*, *SERPINE1* (encoding PAI-1), *IL24*, and negative control siRNAs were as follows: *PRG4*, 5'-CCA CAAAAGCCCUGAUGAA-3'; *SERPINE1*, 5'-GGACAA AACUGGAGAUGCA-3'; *IL24*, 5'-GUGGAUGGGUGCUUA GUAA-3'; negative control, 5'-AUCCGCGCGAUAGUACGU A-3' (19,21,24). Two FlexiTube siRNAs were used against *IL24*, namely, Hs_IL24_5 and Hs_IL24_6 (GeneGlobe IDs SI02638139 and SI02638146, respectively; Qiagen KK).

Western blot analysis. Protein sample preparation was performed as previously described (25). Protein samples were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). The protein samples (10 μg/lane) were then separated using SDS-PAGE (14% gel) and transferred to Hybond ECL nitrocellulose membranes (Amersham; Cytiva). To confirm equal sample loading, the membranes were stained with Ponceau S solution (Sigma-Aldrich; Merck KGaA) for 1-2 min at room temperature. After blocking with 2% bovine serum albumin (BSA; FUJIFILM Wako Pure Chemical Corporation) in Tris Buffered saline with 0.05% Tween-20 (TBS-T; Takara Bio, Inc.; for the antibodies against PAI-1, IL-24 and α-tubulin) or 5% skim milk powder (FUJIFILM Wako Pure Chemical Corporation) in PBS (for all other primary antibodies) at room temperature for 30 min, the membranes were probed with specific antibodies at room temperature overnight. The primary antibodies used were as follows: Purified mouse anti-PAI-1 (1:2,500; cat. no. 612024; BD Biosciences), human IL-24 antibody (mouse monoclonal; 1:2,000; cat. no. K101, GenHunter Corporation), anti-PRG4 antibody (1:2,000; cat. no. AB2200; Merck KGaA), cleaved caspase-3 (Asp175) antibody (1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), poly (ADP-ribose) polymerase (PARP) antibody (1:1,000; cat. no. 9542; Cell Signaling Technology, Inc.), and monoclonal anti-α-tubulin antibody clone B-5-1-2 (1:2,500; cat. no. T-5168; Sigma-Aldrich; Merck KGaA). The membranes were then washed with TBS-T or PBS three times, incubated with 2% BSA in TBS-T with goat anti-mouse IgG (HRP) H&L (1:5,000; cat. no. ab205719; Abcam) or 5% skim milk powder in PBS with goat anti-rabbit IgG (HRP) H&L (1:5,000; cat. no. ab205718; Abcam) at room temperature for 1 h, and washed with TBS-T or PBS three times. The signals were visualized using ECL Prime Western Blotting Detection Reagent (Amersham; Cytiva) and detected with Chemiluminescence CCD Imaging System (AE9300 Ez-Capture MG; ATTO Corporation).

Reverse transcription-quantitative PCR analysis (RT-qPCR). Cellular RNA was extracted from 2645/94 and 1955/91 cells using ISOGEN (Nippon Gene Co., Ltd.). First-strand cDNA synthesis and real-time polymerase chain reaction (PCR) analysis were performed as previously described (21). The relative expression was calculated using the standard curve. The nucleotide sequences of the specific primers for *ACTB* (encoding β-actin) are 5'-GGGAAATCGTGCGTGACATTAAG-3' and 5'-TGTGTTGGCGTACAGGTCTTTG-3' and those for *IL24* are 5'-GTTTTCCATCAGAGACAGTG-3' and 5'-GTAGAA TTTCTGCATCCAGG-3', as previously described (19,26). The mRNA levels of *IL24* were normalized to *ACTB* levels. The PCR amplifications were performed in triplicate.

Chemicals. The proteasome inhibitor, MG-132 (product no. M7449; Sigma-Aldrich; Merck KGaA), was added to the culture medium at a final concentration of 1 μM and incubated at 37˚C for 1, 2, 6 and 24 h. The PAI-1 inhibitor, TM5275 sodium salt (product no. SML1398; Sigma-Aldrich; Merck KGaA), was dissolved with dimethyl sulfoxide at a stock concentration of 50 mM, added to the culture medium at a final concentration of 60 μM and incubated at 37˚C for 48 h.

Statistical analysis. Data are expressed as the mean ± standard deviation of at least three repeats. The individual groups were compared using one-way analysis of variance (ANOVA) followed by Tukey-Kramer's post hoc test. All statistical analyses were performed using Excel 2013 software (Microsoft Corporation), with the add-in software Statcel-the

Useful Addin Forms on Excel-4th ed. (OMS Publishing, Inc.; https://oms-publ.main.jp/main/). P<0.05 was considered to indicate a statistically significant difference.

Results

Proteasomal degradation of IL-24 protein promotes MLS cell survival. The effects of the proteasome inhibitor, MG-132, on MLS-derived 2645/94 cells was first analyzed. At 48 h after transfection with *IL24* siRNA or negative control siRNA, the 2645/94 cells were treated with MG-132 (Fig. 1A). In the negative control siRNA-transfected cells (control), MG-132 induced cell death within 6 h (Fig. 1B), whereas the *IL24* siRNA-transfected cells (IL-24 knockdown) resisted MG-132-induced cell death for at least 6 h (Fig. 1B). Both cells were destroyed 24 h after MG-132 treatment (Fig. 1B). Furthermore, the abundance of IL-24 protein was lower in the IL-24 knockdown cells than in the control cells at 1, 2 and 6 h after MG-132 treatment (Fig. 1C). Similar results were observed at 6 h after MG-132 treatment following transfection with two additional siRNAs targeting different sites in the *IL24* mRNA (Hs_IL24_5 and Hs_IL24_6; Fig. 1D and E). On the other hand, it has been previously demonstrated that IL-24 overexpression supresses the growth of MLS cells (19). Collectively, these results indicated that IL-24 degradation by the ubiquitin-proteasome system contributed to the survival of MLS cells.

PAI-1 knockdown does not affect the mRNA expression of IL24, but enhances the protein expression of IL-24. Next, to determine whether IL-24 participates in the growth suppression mechanism associated with PAI-1 knockdown, knockdown experiments were performed in 2645/94 cells using *SERPINE1* (encoding PAI-1) and *IL24* siRNAs (Fig. 2A, upper panels). *PRG4* siRNA was also used as a positive control for IL-24 induction. The negative control siRNA-transfected cells (control) were used as a negative control. Consistent with previous results (25), PRG4 knockdown suppressed MLS cell growth, whereas PRG4 and IL-24 double knockdown canceled the growth-suppressive effects of PRG4 single knockdown (Fig. 2A, upper panels). Interestingly, double knockdown of PAI-1 and IL-24 also prevented the growth suppression by PAI-1 single knockdown (Fig. 2A, upper panels). These experiments were also performed using another MLS-derived cell line, 1955/91, and similar results were obtained (Fig. 2A, lower panels). Thus, these results indicated that PAI-1 was also involved in the mechanism of IL-24 supression in MLS cells.

To confirm whether PAI-1 knockdown induced IL-24 expression, *IL24* mRNA expression in both cell lines was analyzed using RT-qPCR. In contrast to the PRG4-knockdown results, PAI-1 knockdown did not increase *IL24* mRNA expression (Fig. 2B, left panels). However, the expression of *IL24* mRNA was further downregulated in PAI-1 and IL-24 double-knockdown cells (Fig. 2B, right panels). Thus, although PAI-1 knockdown did not affect *IL24* mRNA, downregulation of IL-24 in PAI-1-knockdown cells may be required for MLS cell survival. Subsequent analysis of IL-24 protein abundance in the cells revealed an increase in IL-24 protein in PAI-1-knockdown cells compared to control cells (Fig. 2C), further supporting the notion that PAI-1 affects IL-24 expression at the protein level.

These findings indicated that the MLS-specific chimeric oncoprotein TLS-CHOP may suppress IL-24 through two separate mechanisms at the mRNA and protein levels, as described in Fig. 3. It is posited that PAI-1 is involved in the degradation of IL-24 protein in MLS cells; however, it remains unclear whether PRG4 transcriptionally regulates or degrades *IL24* mRNA.

PAI-1 may serve as a candidate therapeutic target for MLS treatment. Several pharmacological inhibitors of PAI-1, including TM5275, have exhibited antitumor activity in various tumor cells (27-32). Therefore, it was examined whether TM5275 has tumor-suppressive effects in MLS cells. To confirm the role of IL-24 in TM5275-treated MLS cells, the effects of TM5275 on IL-24 knockdown cells were also evaluated. At 24 h after transfection with *IL24* siRNA or negative control siRNA, the 2645/94 cells were treated with TM5275 (Fig. 4A). Although TM5275 induced cell death within 48 h in the control cells, the IL-24-knockdown cells resisted the effect of TM5275 (Fig. 4B). IL-24 protein expression was induced in the control cells treated with TM5275 (Fig. 4C). Furthermore, the TM5275-treated control cells exhibited high expression of the apoptosis markers cleaved caspase-3 and cleaved PARP, suggesting that PAI-1 inhibition by TM5275 induced apoptosis in MLS cells (Fig. 4C). Collectively, these results provided further evidence that PAI-1 inhibition induced MLS cell death through the induction of IL-24 expression and indicated that PAI-1 may be a candidate therapeutic target for MLS treatment.

Discussion

The present study demonstrated that PAI-1 is a key molecule that supressed IL-24 expression in MLS cells. IL-24 is a unique antitumor cytokine with various tumor-suppressive activities, including the suppression of tumor growth, invasion, and metastasis (33). IL-24 regulates key proteins involved in the regulation of endoplasmic reticulum stress and mitochondrial function, and induces apoptosis with toxic autophagy in diverse malignant tumor cells (33,34). Thus, supression of IL-24 may be crucial for MLS cell survival.

Transcriptional regulation of IL-24 can occur via multiple recognition sites on the *IL24* gene, including those of activator protein-1 (AP-1) and CCAAT/enhancer-binding protein (C/EBP) (35). CHOP is a member of the C/EBP family, and TLS-CHOP, which includes the full-length CHOP, appears to directly affect *IL24* gene expression. However, it was previously demonstrated that the TLS-CHOP downstream molecule PRG4 inhibited *IL24* mRNA expression (21). Furthermore, Madireddi *et al* reported that C/EBP did not supress but rather promoted IL-24 transcription (35). Thus, it was considered that TLS-CHOP does not supress *IL24* mRNA expression directly but indirectly via PRG4 induction. Epigenetic processes such as histone acetylation are also suggested to modulate IL-24 transcription (36). However, no evidence, to date, indicates that epigenetic modifications affect TLS-CHOP-mediated supression of IL-24 transcription.

IL-24 protein abundance has been revealed to be controlled by the ubiquitin-proteasome system (22). The present study revealed that PAI-1 knockdown did not impact *IL24* mRNA

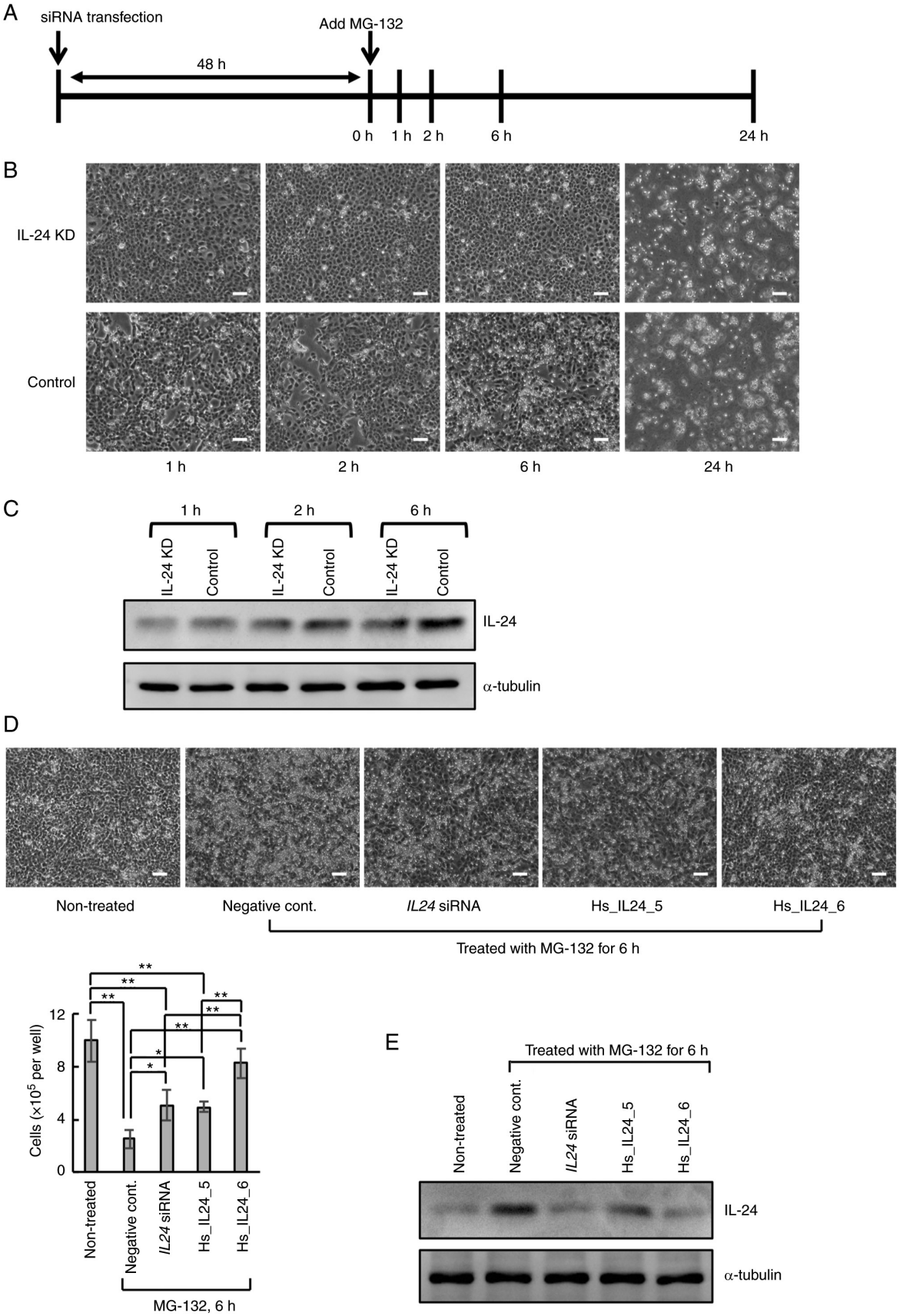

Figure 1. Effects of the proteasome inhibitor, MG-132, on 2645/94 cells. (A) Graphical representation of the experimental timeline. (B) Representative phase-contrast images of 2645/94 cells at 1, 2, 6 and 24 h after the addition of MG-132. Magnification, x40. Scale bar, 100 μm. (C) Western blot analysis of IL-24 expression in 2645/94 cells at 1, 2 and 6 h after the addition of MG-132. α-Tubulin was used as a loading control. (D) Representative phase-contrast images and viable cell counts of 2645/94 cells transfected with siRNA at 6 h after MG-132 treatment. The image and the viable cell count of the 2645/94 cells without siRNA transfection and MG-132 treatment (non-treated) are also displayed. Magnification, x40; scale bar, 100 μm. Data is presented as the mean ± SD. *P<0.05 and **P<0.01. (E) Western blot analysis of IL-24 abundance in the cells shown in D. α-Tubulin was used as a loading control. IL-24, interleukin-24; siRNA, small interfering RNA; KD, knockdown.

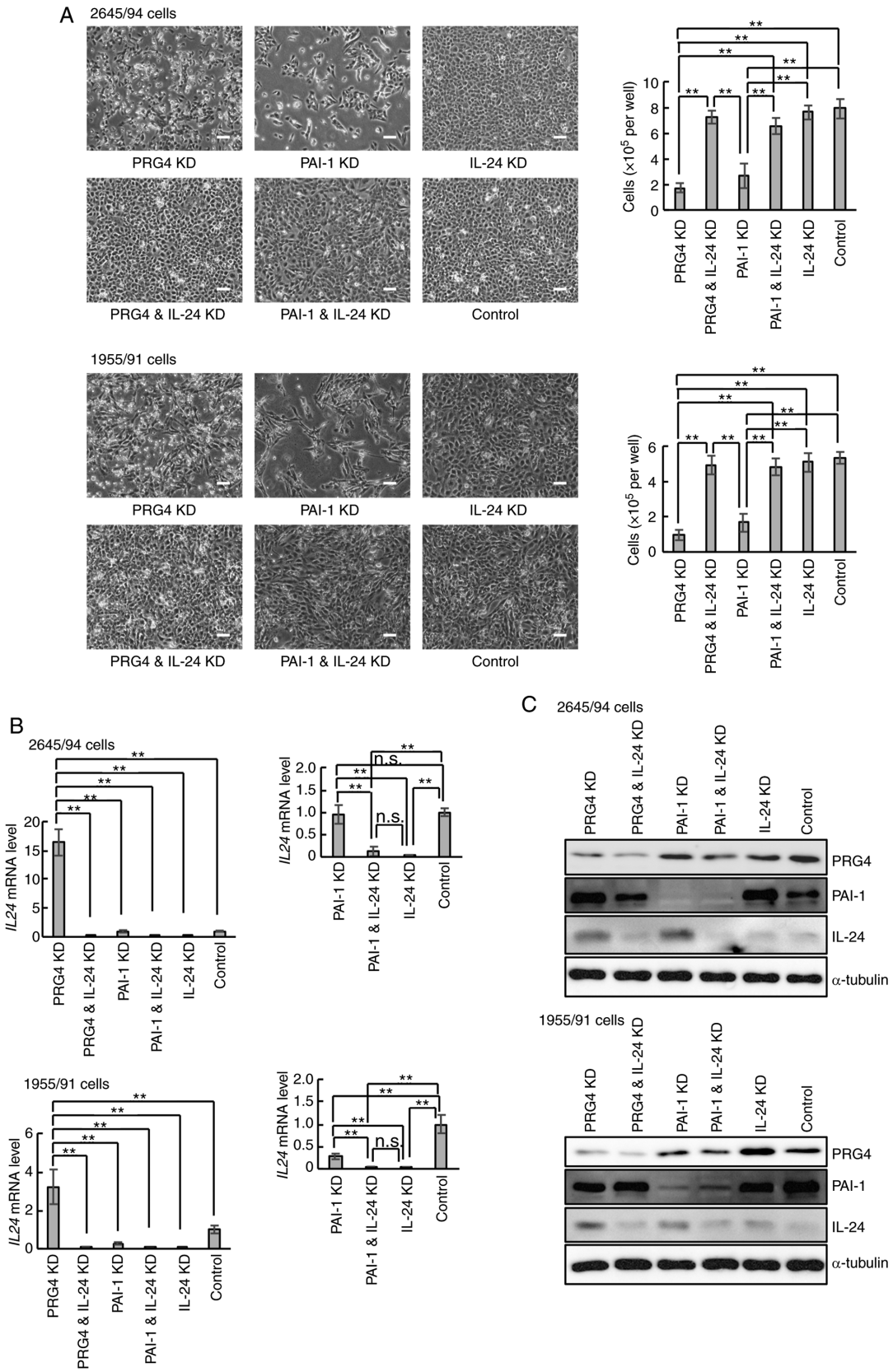

Figure 2. Effects of PAI-1 knockdown on myxoid liposarcoma-derived cells. (A) Representative phase-contrast images and viable cell numbers of 2645/94 and 1955/91 cells at 48 h after siRNA transfection. Magnification, x40; scale bar, 100 μm. Data is presented as the mean ± SD. **P<0.01. (B) Reverse transcription-quantitative PCR analysis demonstrating the mRNA levels of *IL24* in 2645/94 and 1955/91 cells at 48 h after siRNA transfection. Left panels displaying the relative mRNA levels of *IL24* in each cell line. Right panels displaying the relative mRNA levels of *IL24* in each cell line, excluding cells with PRG4 knockdown. The right panel graphs present data extracted from the left panel graphs, respectively, to clarify the differences in the mRNA levels of *IL24* between the indicated cells. The mRNA level of *IL24* in the control cells was arbitrarily set to 1 in the graphical presentation, and all other mRNA signals were normalized to this value. Data are presented as the mean ± SD. **P<0.01; n.s., not significant. (C) Western blot analysis of PRG4, PAI-1 and *IL-24* abundance in 2645/94 and 1955/91 cells at 48 h after siRNA transfection. α-Tubulin was used as a loading control. PAI-1, plasminogen activator inhibitor-1; siRNA, small interfering RNA; *IL24* or IL-24, interleukin-24; PRG4, proteoglycan 4; KD, knockdown.

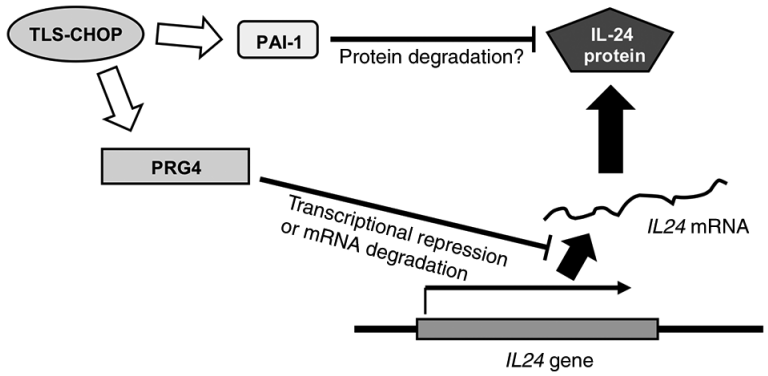

Figure 3. Schematic representation of the mechanisms of IL-24 suppression in myxoid liposarcoma cells. IL-24, interleukin-24; TLS-CHOP, translocated in liposarcoma-CCAAT/enhancer-binding protein homologous protein; PAI-1, plasminogen activator inhibitor-1; PRG4, proteoglycan 4.

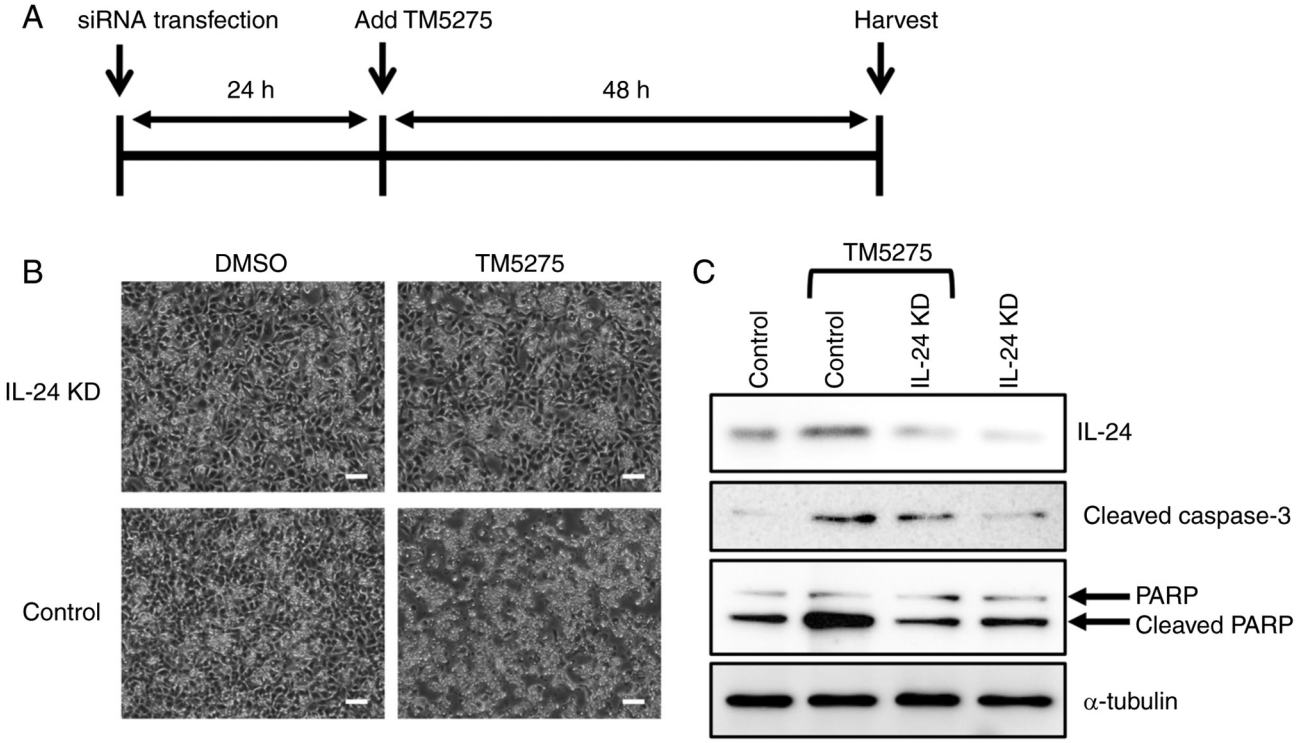

Figure 4. Effects of the inhibitor of plasminogen activator inhibitor-1, TM5275, on 2645/94 cells. (A) Graphical representation of the experimental timeline. (B) Representative phase-contrast images of 2645/94 cells at 48 h after the addition of TM5275. Magnification, x40; scale bar, 100 μm. (C) Western blot analysis of IL-24, cleaved caspase-3, and total PARP in 2645/94 cells at 48 h after the addition of TM5275. α-tubulin was used as a loading control. IL-24, interleukin-24; PARP, poly (ADP-ribose) polymerase; siRNA, small interfering RNA; DMSO, dimethyl sulfoxide; KD, knockdown.

expression but rather increased its protein abundance in MLS cells. Given that PAI-1 reportedly interacts with proteasomes and regulates their activity (37), it was postulated that PAI-1 may directly or indirectly modulate IL-24 degradation through the ubiquitin-proteasome system. In fact, the results of the present study revealed that the proteasome inhibitor, MG-132, induced MLS cell death; while IL-24 knockdown did not fully abrogate these effects, they were diminished. Thus, among numerous substrates for the ubiquitin-proteasome system, IL-24 degradation appears to be important in MLS cells. Collectively, previous studies by the authors (19,21) and the results of the present study indicated that IL-24 was suppressed by TLS-CHOP through two separate mechanisms at the mRNA and protein level in MLS cells.

PAI-1, an inhibitor of urokinase-plasminogen activator and tissue-type plasminogen activator, has multiple functions in diverse pathological processes, including cardiovascular disease and cancer, in addition to its involvement in several biological processes such as fibrinolysis and wound healing (38). However, the role of PAI-1 in apoptosis remains controversial and has not been established in MLS cells (39). The known anti-apoptotic effects of PAI-1 include inhibition of pro-apoptotic mediators (e.g., FasL and caspase-3) and production of anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL) via induction of c-Jun/ERK signaling. The inhibition of cell adhesion to vitronectin by PAI-1 was revealed to exert both pro- and anti-apoptotic activities depending on the condition (40). In the present study, PAI-1 inhibition increased the levels of the apoptotic markers,

cleaved caspase-3 and cleaved PARP, in association with the induction of IL-24 expression and induced cell death in MLS cells. Thus, the results suggest a novel molecular mechanism of PAI-1-mediated anti-apoptotic effects in MLS cells.

PAI-1 is overexpressed in various cancer cells and has been demonstrated to be a promising candidate target for their treatment (23,41). Furthermore, PRG4 is expressed not only in MLS, but also in various sarcomas (42,43). Thus, the mechanisms to suppress IL-24 by PRG4 and PAI-1, respectively, may be important for the growth of different types of cancer cells. As such, disruption of these mechanisms may be a promising therapeutic strategy for numerous cancers. Although the present study has limitations, including the molecular mechanism of the regulation of *IL24* mRNA expression which was not elucidated, and IL-24-knockout MLS cells which were not constructed and analyzed, the results of the present study aid in advancing the current understanding regarding the mechanisms underlying MLS tumorigenesis and progression.

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Availability of data and materials

The datasets shown and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

KO and MK conceived and designed the study. KO performed experiments, analyzed the data and wrote the manuscript. SE assisted with the experimental design, data analysis, and writing of the manuscript. KO and SE confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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