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Targeting of the deubiquitinase USP9X attenuates B-cell acute lymphoblastic leukemia cell survival and overcomes glucocorticoid resistance

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ABSTRACT

Although previous studies attributed a pro-survival role to USP9X in human cancer, how USP9X affects B-cell acute lymphoblastic leukemia (B-ALL) remains unclear. Here, we found that USP9X is overexpressed in B-ALL cell lines and human patients. We investigated the role of USP9X in B-ALL and found that USP9X knockdown significantly reduced leukemic cell growth and increased spontaneous apoptosis, thereby improving survival in immunodeficient mice. These effects are partially mediated by the intrinsic apoptotic pathway, as we found that USP9X-knockdown leukemic cells displayed MCL1 down-regulation, with decreased BCL-2/BCL-XL levels and increased BAX levels. In addition, we demonstrated that USP9X inhibition negatively regulates mTORC1 activity toward its substrate S6K1. Clinically, USP9X inhibition sensitized glucocorticoid-resistant ALL cells to prednisolone; this observation reveals a potential avenue for improving the treatment of drug-resistant relapses. Collectively, our findings suggest that the combination of USP9X targeting and glucocorticoids treatment has attractive utility in B-ALL. This approach represents a potential strategy for promising combination therapies for lymphoid malignancies.

1. Introduction

Acute lymphoblastic leukemia (ALL) is one of the most common malignancies among children, representing 12% of all leukemias and 80% of childhood leukemias [1]. The treatment protocol for various B-ALL subtypes includes an intensified systemic and central nervous system directed chemotherapy regimen that yields cure rates from 15% in 1990 to 80% in recent years [2,3]. However, drug resistance remains a major problem hindering the successful treatment of ALL. One striking example in which resistance to ALL therapeutics predicts outcome is resistance to glucocorticoid (GC) treatment [4]. As long-term GC therapy frequently results in GC resistance via an unclear molecular mechanism, understanding and overcoming GC resistance remains a substantial step towards improving the prognosis of B-ALL. Thus, the development of new safer and more effective agents is necessary to treat relapsed and resistant B-ALL patients.

The ubiquitin-specific protease USP9X is one of the largest deubiquitinases belonging to the Ub-specific protease family. Analogous to phosphatases in kinase signaling pathways, USP9X has been shown to remove ubiquitin moieties, which principally direct target proteins toward proteasomal degradation. A dozen USP9X substrates have been identified, including AF-6, β-catenin, MCL1, Smad4, and survivin [5–9]. Because of its ability to modify a multitude of target proteins, USP9X likely functions in a highly context-specific manner. Several reports have considered USP9X as an oncogene [7,10], whereas conflicting studies have portrayed USP9X as a tumor suppressor [11].

In previous studies, the role of USP9X in B-ALL has not been explored. The underlying mechanisms dependent on USP9X may vary in different cell types and remain unclear. In the present study, we investigated the effect of USP9X on B-ALL. We provide compelling evidence that knocking down USP9X delays the onset of

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leukemia in a xenograft mouse model *in vivo* and effectively inhibits B-ALL cell growth *in vitro*. Clinically, USP9X inhibition sensitizes malignant lymphoid cells to GC-induced apoptosis. We propose a mechanism by which USP9X promotes B-ALL cell survival, validating USP9X as a promising candidate therapeutic target for B-ALL.

2. Materials and methods

2.1. Cell lines and patients

The RS4; 11, Reh, SUP-B15 cell lines were originally obtained from ATCC (Manassas, VA, USA). BV-173 cells were purchased from DMSZ (Braunschweig, Germany). BM specimens were collected from 3 cases of B cell acute lymphoblastic leukemia patients who presented at Tongji Hospital. All leukemia samples were greater than 90% pure leukemic blasts and obtained before chemotherapy. Normal BM samples were obtained from 3 healthy volunteer donors. This study was performed in accordance with the Declaration of Helsinki and under a research protocol approved by the ethics committees of Tongji Hospital.

2.2. Evaluation of engraftments in NOD/SCID mice

After transplantation, mice were returned to their cages and examined daily for general well-being. Engraftment was monitored by weekly blood collections and FACS analysis for human $CD45^+$ ($hCD45^+$) GFP^+ cells. At the first indication of morbidity (>20% weight loss, lethargy and ruffled fur), mice were sacrificed by cervical dislocation. To ascertain the leukemic engraftment levels, the proportions of $hCD45^+GFP^+$ cells in bone marrow (BM), peripheral blood (PB), and spleen were quantified by flow cytometry. The procedures mentioned above were performed as previously described [12]. All studies were performed in compliance with Tongji Medical College Animal Care and Use Committee guidelines.

2.3. Western blotting

Protein sample preparation and Western blots were performed as previously described [13]. Antibodies were purchased from Cell Signaling Technologies, Sigma-Aldrich and Abcam.

2.4. Colony formation assay

Procedures for culturing of leukemic samples in semi-solid methylcellulose and for counting colonies have been previously described [14].

2.5. USP9X silencing by lentiviral-delivered RNA interference

For shRNAs, USP9X-specific target sequence was chosen according to online shRNA tools provided by Invitrogen, using the USP9X reference sequence (Gene Bank Accession No. NM_004652). Double-stranded DNA containing the interference sequence were synthesized according to the structure of a pGCSIL-GFP viral vector (Gikai gene company, Shanghai, China), and then inserted into linearized vector. The target sequence, from 5′ to 3′ was GCTAGAGACCATGAAGATTAT.

2.6. Immunohistochemistry

Paraffin-embedded tissue sections were dewaxed in xylene and subjected to immunohistochemical analysis as previously described [15]. An anti-USP9X rabbit monoclonal antibody (Abcam) and DAB Detection Kit (Streptavidin-Biotin, ZSGB-BIO, Beijing, China) were used in the present study.

2.7. Real-time RT-PCR

Total RNA was extracted from cells with TRIzol (Invitrogen, CA, USA). The expression of gene was quantified using the comparative CT method. Real-time RT-PCR was conducted using a detection system (CFX-96 Real-time PCR System; 97 Bio-Rad, CA, USA) with SYBR Green Mastermix (DBI Bioscience) according to the manufacturer's protocol. The expression level of each mRNA was normalized to that of GAPDH mRNAs, and expressed as n-fold difference relative to the control. The expression of USP9X was analyzed using SYBR green real-time PCR primers: 5′-CTTATCCATAGTGTTGCGAGAT-3′, 5′-GTTTCCCTGCCTGTGCTG-3′.

2.8. Intracellular phospho-staining by flow cytometry

Intracellular phospho-staining was performed as previously described with a few modifications [14]. 5×10^5 BM cells from leukemic NOD/SCID mice were stained with anti-CD45 (PE, BD Pharmingen). 1 ml of single cell suspensions were immediately fixed with 2% paraformaldehyde at RT and subsequently washed, then permeabilized with ice-cold 95% methanol. The cells were then incubated with rabbit anti-p-$S6K1^{Thr389}$ antibody (LifeSpan Biosciences) or rabbit mAb IgG isotype control (LifeSpan Biosciences) labeled by Lightning-Link PE-Cy7 Tandem Conjugation Kit (Innova Biosciences) and then analyzed by flow cytometry on a FACSAria (BD Biosciences).

2.9. Cell viability analysis

The cell viability was detected by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cells (1×10^4/well) were plated in 96-well plates at a total volume of 100 μl per well. 10 μl of CCK-8 solution was added to each well and cultivated for another 4 h at 37 °C, and then the OD value for each well was read at wavelength 450 nm to determine the cell viability on a microplate reader (SpectraMax 190, Molecular Devices, MD, USA).

2.10. Statistics

Unpaired, two-tailed Student's t tests were used for all analyses comparing two experimental groups. Log-rank (Mantel–Cox) test was used to determine *P* values for all Kaplan–Meier survival curve analyses. All data were exported and calculated into Microsoft Excel, and statistical analyses were performed using the GraphPad Prism software (package version 5.0; GraphPad Soft-ware Inc., San Diego, CA, USA). A P value of <0.05 was considered statistically significant.

3. Results

3.1. USP9X is overexpressed in B-ALL cell lines and patients

We investigated the expression levels of USP9X in patients with leukemia using expression data provided in the Oncomine dataset. The mRNA levels in B-ALL cells were substantially higher than those of normal BM mononuclear cells (Fig. 1A). We then examined USP9X protein levels in several B-ALL cell lines (Reh, BV-173, SUP-B15, RS4; 11) and found that USP9X protein levels were significantly upregulated in B-ALL cell lines, whereas the normal mononuclear cells maintained very low levels of USP9X (Fig. 1B, left). To validate our observations in B-ALL cell lines, we examined the protein expression of USP9X in primary BM cells obtained from

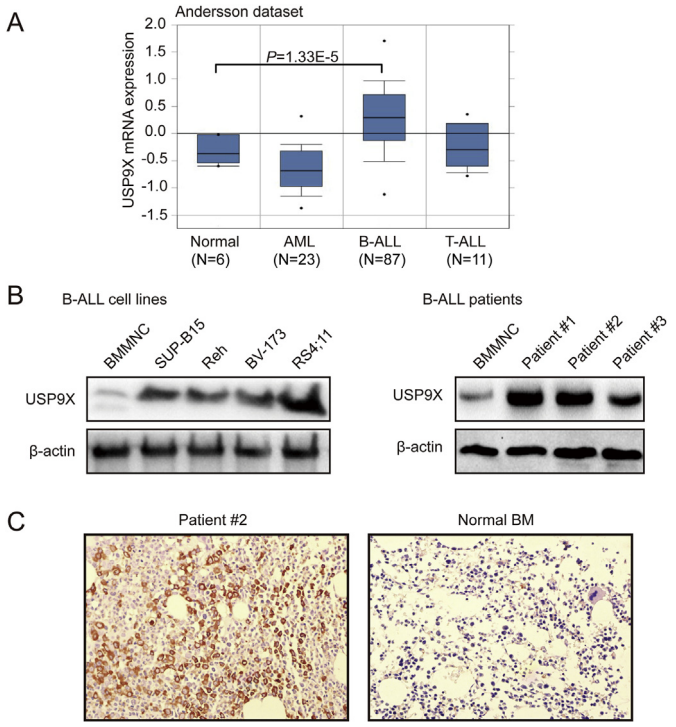

Fig. 1. Overexpression of USP9X mRNA and protein in B-ALL. (A) Relative abundance of USP9X mRNA in the Oncomine Andersson dataset with human genome Swegene 27K microarray data from 127 samples. The log2 median-centered intensities are shown for 4 groups: normal BMMNCs, AML, B-ALL and T-ALL. (B) BMMNCs, B-ALL cell lines (left), and BM cells derived from B-ALL patients (right) were examined by western blotting with USP9X and β-actin antibodies. (C) Representative pictures of USP9X IHC staining (brown) of B-ALL patient #2 BM versus a non-malignant donor (×400).

three different B-ALL patients (Fig. 1B, right). USP9X protein was also highly expressed in leukemic cells from BM specimens of B-ALL patients (Fig. 1C). These data demonstrate that USP9X is overexpressed in B-ALL cell lines or primary leukemia cells from B-ALL patients compared with normal BM mononuclear cells.

3.2. USP9X regulates growth and survival in B-ALL cells in vitro

To address the function of USP9X in B-ALL, we knocked down the expression of USP9X in B-ALL cell lines using lentivirus-delivered shRNA. USP9X mRNA and protein expression were significantly inhibited in RS4; 11 cells (Supplementary Fig. 1). Similar results were found in Reh, BV-173, and SUP-B15 cell lines (data not shown). In the stably transfected cell lines, loss of USP9X resulted in significant growth suppression in B-ALL cell lines and increased spontaneous apoptosis compared with vector controls (Fig. 2A). Additionally, we examined the role of USP9X in primary B-ALL cells. Primary cells transduced with USP9X-1 shRNAs displayed a significant suppression of cell growth and an induction of apoptosis (Fig. 2B). Furthermore, a significantly decreased number of colony forming unit (CFU) blasts was observed (Fig. 2C), indicating that USP9X inhibition negatively affected the colony formation efficiency of primary B-ALL cells. Together with the results from established leukemia cell lines and primary B-ALL cells, these data strongly suggest that USP9X plays a crucial role in B-ALL cells.

We then explored the mechanisms by which USP9X regulated leukemic cell survival. USP9X was recently reported as an MCL1 deubiquitinase and thus as a factor promoting cell growth [7,16,17]. In our study, we found that knocking down USP9X in RS4; 11 cells resulted in the reduced expression of MCL1, BCL-2 and BCL-XL, which are the main anti-apoptotic proteins, and the elevated expression of BAX, which is the main pro-apoptotic protein (Fig. 2D). These results suggest that USP9X promotes leukemic cell survival by regulating BCL-2 family members and that the suppression of USP9X may accelerate MCL1 degradation. Meanwhile, several other proteins have been previously identified to interact with USP9X, including mTOR [18]. To test the idea that USP9X may regulate the function of mTOR, we measured mTOR pathway activity in cells with shRNA-mediated reduced USP9X expression. Transduced RS4; 11 cells followed by serum starvation for 24 h to reduce basal S6K1 phosphorylation levels. The cells were then stimulated for varying lengths of time, and phosphoprotein and total protein levels of S6K1 were measured. As shown in Fig. 2E, knocking down USP9X resulted in significantly decreased S6K1 phosphorylation. These findings confirmed that USP9X is a positive regulator of mTORC1 activity in B-cell lymphoblasts.

3.3. USP9X deficiency suppresses leukemia progression in vivo

To observe the functional consequences of USP9X down-regulation *in vivo*, we injected NOD/SCID mice with shUSP9X-1 or sc-shRNA RS4; 11 cells via the tail vein (Fig. 3A). The appearance of $hCD45^{+}GFP^{+}$ cells that represent the leukemia cells were detected in the PB every 5–10 days, and we observed that USP9X deficiency prevented the propagation of leukemic cells in the PB (Fig. 3B, left). A Kaplan–Meier plot, shown in Fig. 3B (right), suggests the significantly prolonged survival of RS4; 11/shUSP9X-1 recipients compared with the control group (median survival, 38 vs. 47 days; 1×10^7 cells were injected per mouse; $P = 0.0169$). Moreover, eight weeks after the transplant, we sacrificed 4 mice from each group. Flow cytometry confirmed that the down-regulation of USP9X mediated by USP9X shRNA led to reduced frequencies of leukemic cells in the BM as well as the decreased dissemination of leukemia cells into the spleen and lymph nodes (Fig. 3C). Surprisingly, we found that the shUSP9X-1 mice had significantly smaller spleens than the sc-shRNA controls (Fig. 3D). These results provide compelling evidence that USP9X essentially regulates the progress of leukemic RS4; 11 cells and may consequently be a crucial factor for the persistence of leukemia.

To detect the long-term effect of USP9X suppression *in vivo*, we examined protein levels of purified leukemia cells from xenotransplants receiving silenced RS4; 11 cells. The results demonstrated that leukemia derived from USP9X-repressed cells remained stable for a period (Fig. 3E). Furthermore, MCL1 protein levels were also decreased (Fig. 3E), indicating that USP9X could prevent MCL1 degradation via deubiquitylation in the xenotransplant mouse model. In addition, to assess mTORC1 activity *in vivo*, we examined the status of S6K1 in the NOD/SCID mice using flow cytometry. Leukemic cells from the RS4; 11/sc-shRNA group displayed significantly reduced $pS6K1^{Thr389}$ levels, demonstrating that S6K1 activity is decreased in RS4; 11/shUSP9X-1 leukemic mice (Fig. 3F). This result further confirmed that USP9X positively regulates mTORC1 activity *in vivo*.

3.4. USP9X suppression sensitizes ALL cells to GC-induced apoptosis

As USP9X plays a critical role in B-ALL and GC treatment is a cornerstone of ALL therapy [4], we tested whether down-regulation of USP9X induces prednisolone sensitivity. USP9X expression was knocked down in the prednisolone-sensitive ALL cell line RS4; 11. Cells transfected with USP9X-1 shRNAs resulted in a significant suppression of cell growth following treatment with prednisolone for 24 h and 48 h compared with vector controls, as assessed by the CCK-8 assay (Fig. 4A). In addition, we investigated the induction of apoptosis by prednisolone treatment in transduced RS4; 11 cells

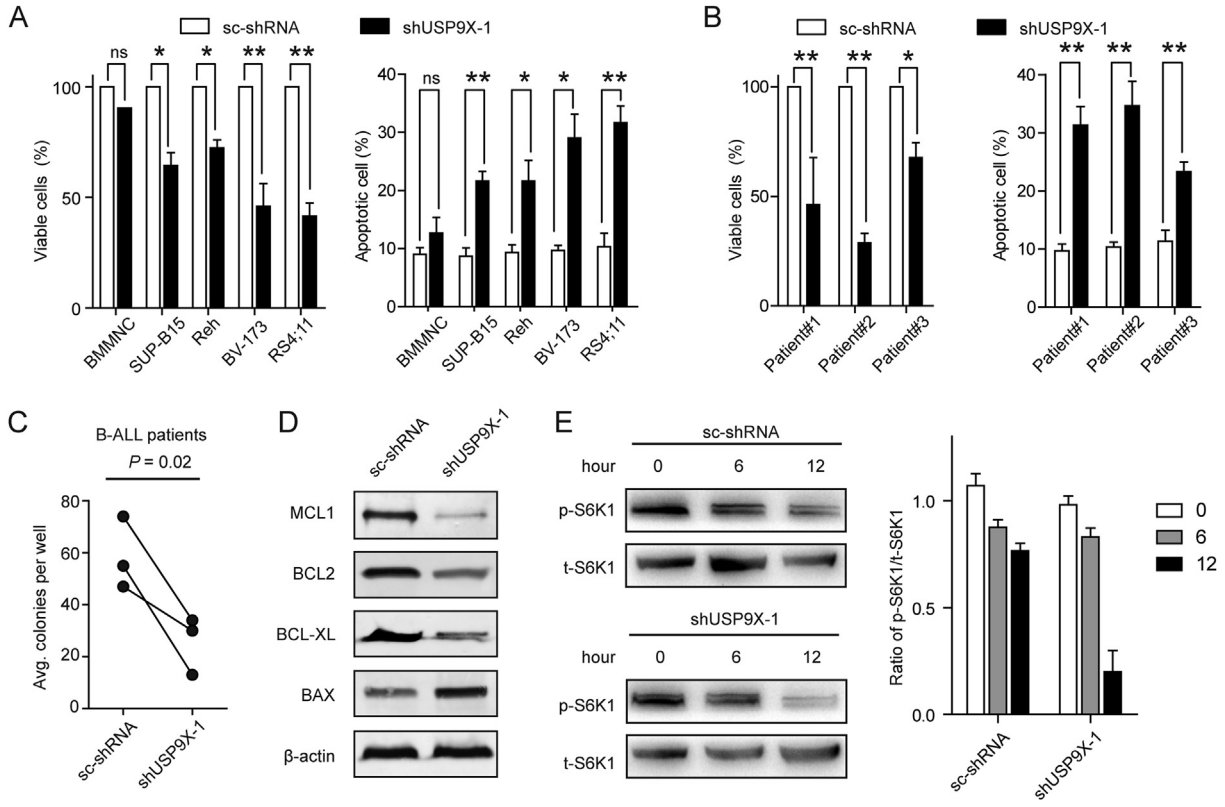

Fig. 2. Stable shRNA-mediated knockdown of USP9X leads to the growth inhibition of B-ALL cells *in vitro*. (A–B) Analysis of cell viability and apoptosis for BMMNCs from a non-malignant donor, B-ALL cell lines and B-ALL patient samples (#1, #2, and #3) 72 h after infection with shUSP9X-1. (C) Transduced patient samples #1, #2, and #3 cells were placed in methylcellulose supplemented with human cytokines. Graph represents the calculation of colonies formed after 12 days of culture. (D) Western blotting was used to measure the levels of apoptosis-related proteins (MCL1, BCL2, BCL-XL and BAX) after USP9X knockdown in RS4; 11 cells. (E) Transduced RS4; 11 cells were serum starved for 24 h followed by stimulation with media containing insulin and serum for the indicated times. Phospho-S6K1 and total S6K1 levels were detected using western blot analysis, and images were quantitated using ImageQuant. Phospho-S6K1 levels were normalized to total S6K1 levels. Representative Western blot (left) results and their quantitation (right) are shown. The quantitation of phospho-S6K1 levels for three biological replicates at various time points (0, 6, and 12 h) are shown. *, $P < 0.05$; **, $P < 0.005$.

using the ANNEXIN-V/7AAD flow cytometric assay. We observed that apoptotic cell death induced by prednisolone was significantly increased in cells transfected with USP9X-1 shRNA compared with those transfected with control shRNAs after treatment with prednisolone for 24 h and 48 h (Fig. 4B). To further assess the role of USP9X in GC resistance, we knocked down USP9X expression in the prednisolone-resistant ALL cell line Reh. Surprisingly, cell growth inhibition (Fig. 4C) and increased cell apoptosis (Fig. 4D) were observed in Reh cells transduced with USP9X-1 shRNA, whereas shRNA-control cells revealed no change after prednisolone treatment. These data suggest that USP9X down-regulation sensitized both GC-resistant and GC-sensitive malignant human lymphoid cell lines to GC-induced apoptosis, demonstrating that USP9X inhibition can potentiate GC-induced cell death.

4. Discussion

In some solid tumors, USP9X has been proposed as a therapeutic target [7]. However, the reports investigating USP9X in leukemia are relatively rare. Here, we sought to explore the role of USP9X in leukemia based on the observation that USP9X was overexpressed in B-ALL. We asked whether targeting USP9X expression was possible and then revealed the clinical significance of our findings. We used a xenograft mouse model to analyze B-ALL progression following USP9X down-regulation and demonstrated that USP9X plays a critical role in governing the progress of leukemia. USP9X-knockdown mice showed reduced disease burden and extended lifespans when transplanted with RS4; 11 cells. Consistent with the *in vivo* results, we confirmed decreased leukemic growth and colony formation *in vitro*. Moreover, we identified that the shRNA-mediated inhibition of USP9X promotes GC-induced apoptosis. Therefore, we proposed that an underlying mechanism could exist to make targeting USP9X useful for therapy in B-ALL.

Recent studies have demonstrated that USP9X is a deubiquitinase for the pro-survival protein MCL1. The deubiquitinase USP9X removes polyubiquitin chains, targeting MCL1 for proteasomal degradation [7]. In addition, the inhibition of USP9X using siRNAs increases the sensitivity of leukemic cells to imatinib [10]. To determine the effect of USP9X inhibition on B-ALL cell survival in our experiments, we used a lentivirus strategy and demonstrated that USP9X-knockdown leukemic cells displayed MCL1 down-regulation, thereby suppressing cell survival *in vivo* and *in vitro*. Meanwhile, USP9X down-regulation is associated with decreased BCL-2/BCL-XL levels and increased BAX levels, indicating that USP9X may play a critical role in cell apoptosis mediated by BCL-2 family proteins. Furthermore, we further verified that knocking down USP9X decreased mTORC1 activity toward its substrate, S6K1, both *in vivo* and *in vitro*. mTORC1 is an important serine/threonine kinase that responds to the extracellular environment to regulate a number of cellular processes, including cell growth, proliferation, and differentiation [19]. In the context of B-ALL, the deubiquitinase USP9X acts as a positive regulator of mTORC1, which may be another contributing factor that concomitantly leads to the inhibition of cell growth.

The outcome in B-ALL has moderately improved in the last decade by novel avenues such as monoclonal antibody therapy,

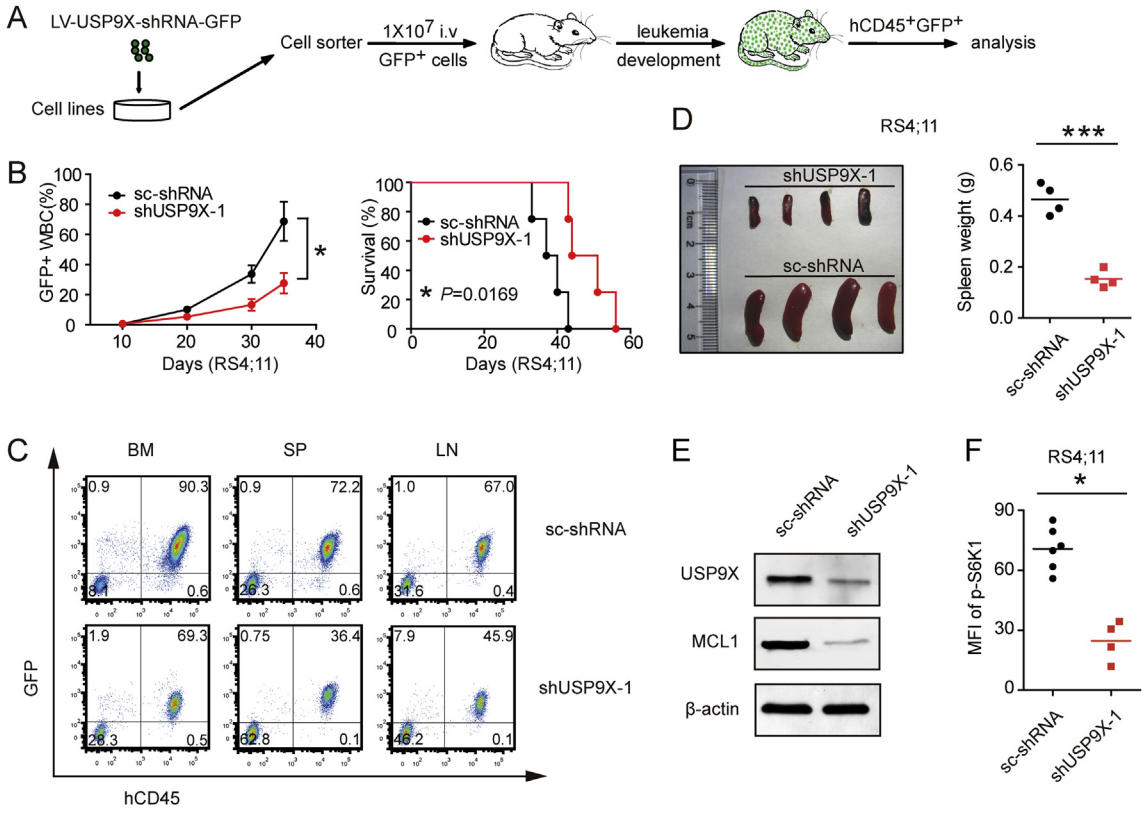

Fig. 3. Knocking down USP9X decelerates B-ALL development in the NOD/SCID mouse model. (A) The lentiviral transduction procedure and experimental design. (B) Left panel, the different dynamics of GFP$^+$ leukemia cells appearing in the PB in recipient mice (n = 7 per group) transplanted with shRNA-transduced RS4; 11 cells. Data represent the mean percentages ± SD. Right panel, the survival of recipient NOD/SCID mice (n = 10 per group) after the transplant of RS4; 11 cells. Log-rank statistical analysis was performed to calculate *P* values. (C) Representative results from the FACS analysis of recipient NOD/SCID mice receiving shUSP9X-1 RS4; 11 cells compared with sc-shRNA control cells. Staining of the cells from the BM, spleen and lymph nodes was performed using PE-conjugated anti-human CD45 antibodies and GFP for the detection of human RS4; 11 cells. (D) Gross anatomical view of spleens recovered from mice transplanted USP9X-1-knockdown cells. The spleens (left) and their weights (right, mean ± SD) are shown for the USP9X-1-knockdown leukemic mice and sc-shRNA-treated leukemic mice (n = 4 per group). (E) Cells were collected from the shUSP9X-1 and sc-shRNA leukemic mice and then subjected to flow cytometry to detect phospho-S6K1. The FACS data are reported as mean fluorescence intensities (MFIs), as analyzed with Flowjo software. (F) Western blotting showing USP9X and MCL1 protein levels of hCD45$^+$GFP$^+$ leukemic cells sorted from shUSP9X-1 and sc-shRNA leukemic mice. *, $P < 0.05$; **, $P < 0.005$.

tyrosine kinase inhibitors specific for Ph/bcr-abl-positive ALL, use of the hypomethylating agent decitabine and chimeric antigen receptor therapy [20]. However, GCs are the backbone drugs used in multi-drug ALL therapy. Relapsed ALL patients always exhibit GC resistance to other anti-leukemic agents. To date, various mechanisms have been described that could sensitize leukemic cells to prednisolone *in vitro*, including repressing the prednisolone interconverter 11β-HSD, downregulating the calcium scavengers S100A8/S100A9, inhibiting the voltage-dependent channel hERG1, downregulating anti-apoptotic MCL1 and upregulating pro-apoptotic BIM [21–25]. In our study, we surprisingly found that knocking down USP9X sensitized malignant human lymphoid cell lines to GC-induced apoptosis.

GC-induced apoptosis is modulated by the intrinsic apoptotic pathway, involving the loss of mitochondrial transmembrane potential and the release of apoptotic factors from the mitochondria. BCL-2 family proteins are crucial in regulating apoptosis induced by multiple cellular stresses, including exposure to GCs [26]. The previous study identified that MCL1 is preferentially expressed in primary ALL cells with resistance or sensitivity to prednisolone and that MCL1 is a key anti-apoptotic protein governing GC resistance. The observations that MCL1 overexpression inhibits GC-induced apoptosis and that shRNA-mediated down-regulation of MCL1 expression sensitizes cells to GC-induced apoptosis have been reported [27]. On the basis of the importance of USP9X in controlling MCL1 levels, we knocked down USP9X in ALL cell lines and found that cells transduced with USP9X shRNAs demonstrated increased apoptosis following prednisolone treatment not only in the prednisolone-sensitive RS4; 11 cell line but also in the prednisolone-resistant Reh cell line. Furthermore, it has been verified that inhibition of the anti-apoptotic BCL-XL sensitized ALL cells to GC-induced apoptosis [28]. We demonstrated that USP9X reduction is correlated with decreased BCL-2/BCL-XL levels, providing additional evidence that the alteration of critical control points in the intrinsic apoptotic pathway represents an attractive potential mechanism to explain how USP9X controls the GC sensitivity of ALL cells.

The present data clearly demonstrate an indispensable role for USP9X in B-ALL and point at its potential as a therapeutic target during GC-based treatment regimens. Our findings show USP9X sensitizes human ALL cell lines to GC-induced apoptosis via modulation of the intrinsic apoptotic pathway. This finding warranting further studies to identify more specific inhibitors to target USP9X and thereby reverse the apoptotic resistance correlated with USP9X activity and MCL1 protection. Taken together, USP9X is a potential target to therapeutically inhibit leukemic cell survival and to overcome drug resistance in ALL. It remains to be determined whether USP9X inhibition represents a more global strategy to be used in other forms of cancer.

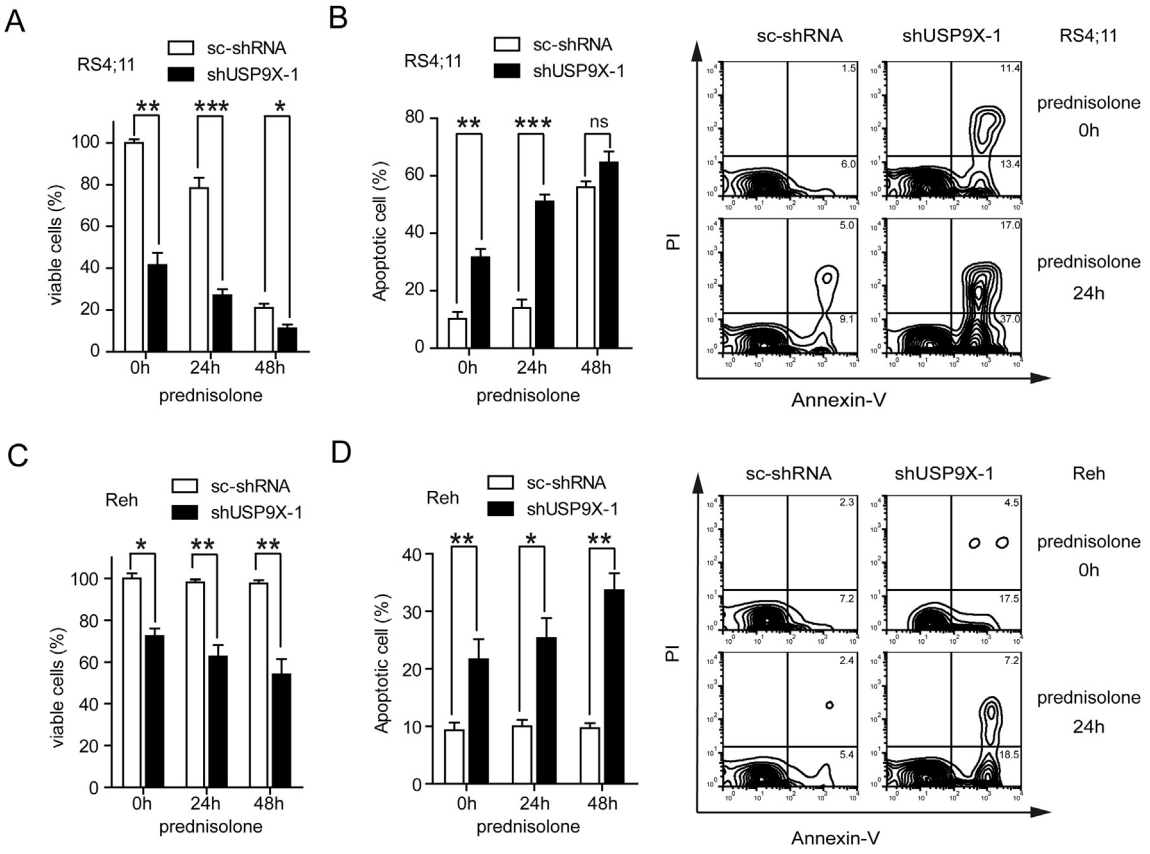

Fig. 4. Knocking down USP9X sensitizes prednisone-sensitive and prednisone-resistant ALL cells to glucocorticoid-induced apoptosis. (A and C) The percentage of viable cells was determined in transfected ALL cells (prednisolone-sensitive RS4; 11 and prednisolone-resistant Reh) 0 h, 24 h and 48 h after treatment with 1.0 μg/ml of prednisolone. (B and D) Apoptosis was monitored on the transfected ALL cells for 24 h and 48 h with or without 1.0 μg/ml of prednisolone treatment using Annexin V/PI staining and FACS. Left, shown are quantifications of apoptotic cells as percent of Annexin V-positive cells. Right, representative flow cytometric profiles of prednisolone induces cell apoptosis in transfected ALL cells after 0 h and 24 h treatment. Data shown are results derived from 3 independent RNA interference experiments. *, $P < 0.05$; **, $P < 0.005$.

Conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.115.

Transparency document

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