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FoxO1 controls the expansion of pre-B cells by regulating the expression of interleukin 7 receptor α chain and its signal pathway

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ABSTRACT

Forkhead box O1 (FoxO1) has a crucial role in the early B cell development. To understand the functional importance of *FoxO1* gene in the early B cell expansion, we established a *FoxO1* knockdown model using 70Z/3 pre-B cell line. The *FoxO1* knockdown 70Z/3 cells (70Z/3-KD cells) showed the down-regulated expression of interleukin 7 receptor α chain (IL-7Rα). Moreover, the signaling via IL-7Rα was significantly attenuated in the 70Z/3-KD cells, and this alteration was fully rescued by re-expression of *FoxO1* gene. Compared to the mock cells, loss of FoxO1 reduced the growth rates in the 70Z/3-KD cells, and was fully rescued by reintroduction of *FoxO1* gene. The expansion of pre-B cells ($CD45R^+CD43^-$ fraction) was also reduced by the knockdown of *FoxO1* gene. Indeed, FoxO1 induces accumulation in the p27-mediated G0/G1 phase arrest in 70Z/3 cells. FoxO1 bound to the *Il7ra* locus specifically and regulate the IL-7Rα transcription. In conclusion, FoxO1 regulates the expansion of pre-B cells by regulating the expression of IL-7Rα and its signal transduction.

1. Introduction

B cells are derived from hematopoietic stem cells. During development, the common lymphoid progenitor begins the process of specification and eventual commitment to the B lineage, which is governed by a network of transcriptional factors including E2A, EBF1 and Pax5, as well as the necessary cytokines, such as interleukin 7 (IL-7), SCF, and Flt-3 L, these modulate B cell survival, growth and development [1,2]. However, the cell-intrinsic factors responsible for the early B cell survival and the manner in which they manifest changes in early B cell populations remain poorly defined.

Forkhead box O (FoxO) family transcription factors are associated with multiple metabolic reactions by regulating the expression of numerous genes involved in apoptosis, cell cycle progression, nutrient availability, DNA repair, stress and angiogenesis [3,4]. FoxO1 contains 4 functional domains, including nuclear localization signal domain (NLS), nuclear export signal (NES), transactivation domain (TA) and Forkhead domain (FKH) [5]. FoxO1 binds to two consensus sequences, including 5′-GT AAA (T/C) AA-3′, which is known as the Daf-16 binding element (DBE), and 5′-(C/A) (A/C) AAA (C/T) AA-3′, which is the insulin-responsive sequence (IRE) [6]. Upon antigen or cytokine stimulation, they are rapidly phosphorylated and deactivated in a PI(3)K-dependent manner, and then FoxO1 translocate to the cytoplasm and result in proteasomal degradation, whereas cytokine withdrawal causes their dephosphorylation and activation [7].

In comparison to other FoxO family members, FoxO1 plays a superior role in the regulation of early stages of B cell differentiation [8,9]. Conditional deletion of FoxO1 resulted in impaired B cell development at the early pro-B and small pre-B cell stages, due to a failure to express IL-7 receptor α (IL-7Rα) [10]. FoxO1 promotes differentiation, proliferation, survival and immunoglobulin gene rearrangement in B cells [8]. Nuclear FoxO1 promotes lymphomagenesis in germinal center B cells [11]. Deletion of FoxO1 in peripheral B cells led to fewer lymph node B cells due to reduced L-selectin expression, and failed class switch recombination [10].

Due to the early B cell arrest of FoxO1 deficient mice, the role of

Abbreviations: 70Z/3-KD cells, FoxO1 knockdown cells; 70Z/3-KD-Re cells, FoxO1 restored 70Z/3-KD cells; BrdU, bromodexyuridin; CDK, cyclin-dependent kinase; ECRs, evolutionarily conserved noncoding regions; FBS, fetal bovine serum; FoxO1, Forkhead box O1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IL-7, interleukin 7; JAK, Janus Associated Kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; STAT5, signal transducer and activator of transcription-5; ORF, open reading frame

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FoxO1 gene in early B lineage development is difficult to study in murine system. To gain insight, we established a novel *FoxO1* knockdown 70Z/3 pre-B cell line (70Z/3-KD) using retro virus system containing FoxO1-siRNA and a FoxO1 restored (70Z/3-KD-Re) cells. Our results provide evidence that FoxO1 maintains the pre-B cells proliferation and survival with signal transduction via IL-7Rα.

2. Materials and methods

2.1. Mice

Mice (C57/B6) were purchase from the specific-pathogen-free laboratory animal facility of Dalian Medical University and maintained in a room illuminated for 12 h (hr) (08:00 to 20:00) and kept at 24 ± 1 °C with free access to food and water in a specific-pathogen-free environment. The method of euthanasia (asphyxiation by CO_2 and cessation of breathing) is an approved AVMA method of euthanasia. All the animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the Ethical Committee of Care and Use of Laboratory Animals at Dalian Medical University (AEE17013).

2.2. Antibodies

FITC-labeled anti-IgM (II/41), PE-labeled anti-CD43 Ab (S7), PE-Cy5.5 labeled anti-IgD (11–26), and APC-labeled anti-CD45R Ab (RA3-6B2) were obtained from e-Bioscience; anti-FoxO1 (76E10), anti-JAK1, anti-p-JAK1 (Tyr1022/1023), anti-STAT5, anti-p-STAT (Thy694), anti-p21 (2946) and anti-p27 (2552) Abs were obtained from Cell Signaling Technology (Danvers, MA); anti-IL-7Rα Ab (CD127, ab155755) and FITC-labeled anti-IL-7Rα Ab (ab33704) were from Abcam (Cambrige, Mass); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-25778) and anti-lamin A Ab (H-102; sc-20680) were from Santa Cruz; horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG was from ICN Pharmaceuticals, Inc. (Arora, OH).

2.3. Transient transfection of mouse FoxO1 siRNA

70Z/3 cells (pre-B lymphoma cell line) were obtained from Prof. Katsuhiko Ishihara. FoxO1 siRNAs were transfected with *TransIT*-TKO transfection reagent (TAKARA Bio. Inc.) to 5×10^5 cells in 6-well plates. The siRNAs were designed to form 19 bp dsRNA with 2 thymine overhangs at both 3′ ends. Three targeting siRNA sequences for the *FoxO1* (NM_019739.3) used: No.1 (sense: 5′-GCU GUC AGC ACC GAC UUU ATT-3′, antisense: 3′-TTC GAC AGU CGU GGC UGA AAU-5′) (652–670), No. 2 (sense: 5′−CCG CCA AAC ACC AGU CUA ATT-3′, antisense: 3′-TTG GCG GUU UGU GGU CAG AUU-5′) (1681–1699), and No. 3 (sense: 5′-GCA GCC AGG CAU CUC AUA ATT-3′, antisense: 3′-TTC GUC GGU CCG UAG AGU AUU-5′) (1979–1997).

2.4. Establishment of FoxO1 gene knockdown (70Z/3-KD) cells and FoxO1 restored cells

The retrovirus vector carrying FoxO1-shRNA was constructed as follows: A 21-nucleotide sequence (1979–1997) of the FoxO1 cDNA was inserted into the pSINsi-mU6 cassette vector (TAKARA Bio. Inc.). Sense: 5′-GATCC (BamH I) GCA GCC AGG CAT CTC ATA A TTCAAG AGA (hairpin loop) TTA TGA GAT GCC TGG CTG CTTTTTTT AT (Cla I)-3′ and antisense: 5′-CGAT (Cla I) AAAAAAAGC AGC CAG GCA TCT CAT AAT TCTCTTGAA (hairpin loop) TTA TGA GAT GCC TGG CTG C CG (BamH I)-3′ (For FoxO1-silencing). Recombinant retrovirus vector, pGP vector (gag-pol) and pE-eco vector were co-transfected to HEK293 cells to generate the recombinant retrovirus particle. 70Z/3 cells were then infected by recombinant retroviruses including the FoxO1-shRNA sequence, and the geneticin (G418)-resistant clones were selected. The 70Z/3 cells stably transfected with the FoxO1-shRNA are referred to as "70Z/3-KD".

For establishment of FoxO1 restored cells, ORF of FoxO1 gene was cloned into pLHCX vector (Clontech). FoxO1 restored 70Z/3-KD (70Z/3-KD-Re) cells were established by the transfection of pLHCX-FoxO1 into 70Z/3-KD cells. To construct pLHCXsi-mU6-FoxO1 expression vectors resistant to the FoxO1-shRNA in the 70Z/3-KD cells, we introduced 3 point mutations into the FoxO1 1979–1997 region of pLHCXsi-mU6-FoxO1 without change of the original amino acid residues.

2.5. Cell culture

70Z/3 derivative cells or pre-B cells ($CD45R^+CD43^-$) sorted on a FACSAria from bone marrow cell suspension were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) including 5% fetal bovine serum (FBS), 10 mM HEPES, 2 mM L-glutamine (Life Technologies) and 5×10^{-5} M β-mercaptoethanol. When indicated, cells were treated with 10 ng/ml recombinant mouse IL-7 (rIL-7) (Sigma).

2.6. Cell lysis

Cells were lysed by sonication with cell lysis buffer [150 mM NaCl, 50 mM Tris−HCl, 1% Triton-X 100, 5 mM, 0.1 mM Na_3VO_4, ethylene diamine tetraacetic acid (EDTA), 10 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT)]. For nuclear protein expression, cells lysates were prepared using nuclear extraction reagents (Pierce, Rockford, IL, USA). The cell lysate was centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentration was measured with bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL).

2.7. Immunoprecipitation

Cell lysate were incubated with antibody overnight with gentle rocking and then added to 20 µl of protein G-sepharose (Amersham Biosciences) for another incubation of 2 h. The immunoprecipitate was washed three times with lysis buffer.

2.8. Western blots

Five µg of protein was electrophoresed on 10% polyacrylamide gel using Mini Protean II electrophoresis tanks (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millopore, Billerica, MA). The membranes were blocked for 2 h with 5% skim milk in TBS-T (10 mM Tris−HCl pH = 7.5 and 0.1% Tween 20) and incubated with the indicated primary antibody. After washing, the membranes were then incubated with an appropriate HRP-conjugated secondary antibody, and visualized by chemiluminescence using an ECL kit (Pierce, Rockford, IL).

2.9. Flow cytometry analysis

Cell suspension prepared from the bone marrow was blocked with anti-CD16/CD32 (2.4G2) mAb, and then incubated with indicated fluorochromeconjugated antibodies in PBS including 2 mM EDTA and 0.01% NaN_3 for 15 min. Flow cytometry was carried out on a FACSCalibur (Becton Dickinson, Mountain View, CA), and the results were analyzed with the CellQuest (Becton Dickinson).

For the cell sorting, BM cells prepared were incubated with FITC-IgM, PE-Cy5.5-IgD, PE-CD43 and APC-CD45R. Then subpopulations were sorted with a FACStar Plus (Becton Dickinson) instrument. Cell purity was routinely over 90%.

2.10. Cell cycle distribution assay

Cells were fixed in 75% ethanol for 2 h at 4 °C and then were rehydrated with PBS and incubated with 500 µl (200 µg/ml) propidium iodide (PI) solution for 30 min at RT. For each sample the percentages of cells in the G0/G1, S and G2/M phases of the cell cycle were calculated using a flow cytometer with FACS software. For each sample, 10,000 fluorescence signals were measured.

2.11. Colony assays

The growth ability of pre-B cells dependent on rIL-7 were evaluated by clonable pre-B cells assay. The $CD45R^+CD43–$ pre-B cells from BM were cultured into methylcellulose media containing 10 ng/ml rIL-7. After culture for 7 days, the colony of pre-B cells was observed under a microscope. The frequencies of pre-B cells were then calculated according to the formula; frequency = {ln [T/(T-P)]}/N. T; number of total wells, P; number of wells containing a pre-B cell colony and N; inoculated cell number per well.

2.12. Apoptosis assay

Cells (5×10^5) were 5 incubated with 5 µl annexin V-FITC (Beyotime Institute of Biotechnology, Haimen, China) and 10 µl PI (20 µg/ml) solution at RT for 30 min. The results were analyzed by using a flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.13. Cell viability assay

Cell viability assay was measured by bromodexyuridin (BrdU) assay kit (CycLex Co., Ltd., Nagano, Japan). In brief, cells were cultured in the 96-well plates and exposed to 10 µm BrdU for 6 h at 37 °C. The cells were then fixed with Denaturing solution for 30 min at RT. After fixing, the wells were added 50 µl of anti-BrdU monoclonal Ab for 1 h at RT. After washing, the cells were incubated with 50 µl HRP-conjugated anti-mouse IgG for 1 h. Finally, the cells incubated with 50 µl of Substrate reagent for 15 min, and the absorbance was measured at 450 nm.

2.14. Real-time PCR

Total RNA was isolated using the RNAiso plus (Takara BioInc., Shiga. Japan) and the first strand of cDNA synthesis were performed using the PrimeScript™. The mRNA leaves were observed by quantitative PCR with SYBR Premix Ex Taq, Rox plus (Takara. Biotechnology CO., Dalian. Chain) and primers as listed in supplementary Table I. Using the expression levels of each candidate genes were calculated by the 2-ΔΔCt method, using GAPDH as the internal normalized control with the same calibrator. Each experiment was performed independently in triplicate.

2.15. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (CHIP) was carried out using a chromatin immunoprecipitation kit (17–295; Upstate Biotechnology). In brief, 1×10^7 cells were fixed with 1% formaldehyde with agitation for 10 min at RT. Cells were lysed by sonication with a digital Sonifier 250 (six 10-s pulses; Branson Ultrasonics). Cell lysates were precleared with salmon sperm DNA-protein A agarose for 2 h at 4 °C, and then incubated with either anti-FoxO1 (H-128) Ab or rabbit IgG. Protein-DNA immune complexes were purified with protein A agarose beads. After treatment with proteinase K, DNA was extracted with phenol-chloroform, and precipitated with 100% ethanol for 2 h at −20 °C, washed and resuspended in Tris-EDTA buffer. Immunoprecipitates and input fraction were analyzed in duplicate by QPCR. The primers for *Il7ra*-ECR (-3765 to -3575) are 5′-ACC TCA TCA GCC TTT CAT GG-3′ and 5′-ATC CCC TGA GCA AAC TAG CAA-3′ (190 bp); for *Il7ra* promoter (-325 to -90) are 5′-GCA GTT AAG TTC AGG AGC TTC AGG-3′ and 5′-GAA GCA CGG TTG TAT GTG CAA GTG-3′ (235 bp); for *Il7ra* exon VIII are 5′-CTG GAC TGC CAA TTC ATG AGG TG-3′ and 5′-TCT CTG TAG TCA GGG GAC CTA GAG-3′ (265 bp).

2.16. Statistical analysis

Statistical analyses are performed using Student's t-test, and the data are expressed as mean ± standard deviation (SD). Data analyses were performed with the statistical software package SPSS 13.0. The statistical significance was assessed by one-way analysis of variance (ANOVA). A P value less than 0.05 was statistically significant.

3. Results

3.1. FoxO1 is preferentially expressed in pre-B cell subsets

B lymphopoiesis takes place in niches within the bone marrow, which provide the structure for development. To investigate FoxO1 relative functional importance during B cell development, we first quantified its population in B cell subsets, $CD45R^+CD43^+IgM^-IgD^-$ (enriched pro-B), $CD45R^+CD43^-IgM^-IgD^-$ (enriched pre-B), $CD45R^+IgM^-$, $CD45R^+IgM^+$ (enriched immature-B) cells, sorted from bone marrow by flow cytometric analyses (Fig. 1A). FoxO1 is expressed in all B cell subsets analyzed. Indeed, real-time PCR analyses showed a significant up-regulation of FoxO1 mRNA from pro-pre B cell transition in the bone marrow (Fig. 1B). Moreover, the protein expression of FoxO1 was increased at the stage pre-B cells (Fig. 1C). Consistent with these results, conditional deletion of FoxO1 resulted in impaired B cell development at the early pro-B and small pre-B cell stages [10] and FoxO1 activation begins during the pre-pro-B cell phase [12]. These observations support the idea of an essential role for FoxO1 in early B cell development.

3.2. Loss of FoxO1 gene reduced the expression of IL-7Rα

FoxO1 transcriptionally up-regulates IL-7Rα expression, controlling proliferation and apoptosis of pro-B cells after IL-7 stimulation [10]. To explore the biological function of FoxO1 in pre-B cells, we used a retrovirus-mediated short hairpin RNA expression system to knockdown *FoxO1* gene expression in 70Z/3 cells. First, we designed specific FoxO1-siRNA fragments complementary to different regions of *FoxO1* open reading frame (ORF) for the knockdown of *FoxO1* gene expression. The sequences of the siRNA correspond to nucleotides 652–670 (siRNA1), 1681–1699 (siRNA2) and 1979–1997 (siRNA3) of mouse FoxO1 mRNA. In real-time PCR analysis, the siRNA3 fragment showed a stronger inhibitory effect than another siRNAs (Fig. 2A).

It is important to assess the role of FoxO1 under conditions where the effect of endogenous FoxO1 is completely eliminated. To mimic the extremely down-regulated expression level of FoxO1, we designed a retrovirus expression system using a pSINsi-mU6 cassette vector inserted with FoxO1 siRNA3 and established stable *FoxO1* knockdown clone, referred to as 70Z/3-KD cells. The introduction of FoxO1 siRNA almost completely suppressed the expression of FoxO1 mRNA and FoxO1 protein in 70Z/3-KD cells (Fig. 2B, and C). To address the underlying molecular function of FoxO1 in the pre-B cell differentiation, we examined the expression pattern of genes essential for B cell development and proliferation by real-time PCR. We found that the FoxO1 mRNA expression in 70Z/3-KD cells was remarkably decreased by a decrease of 75% relative to 70Z/3 cells. Moreover, expression of FoxO1 target genes, such as IL-7Rα, p21 and p27 mRNAs was also significantly downregulated (Fig. 2D). Early B cell development is controlled by a hierarchical regulatory network that induces several key transcription factors, such as E2A, EBF and Pax5 [13]. E2A (encoded by Tcfe2a) and EBF1 (encoded by Ebf1) both bind to their respective loci on the FoxO1 promoter to enhance FoxO1 expression [12]. No obvious changes were

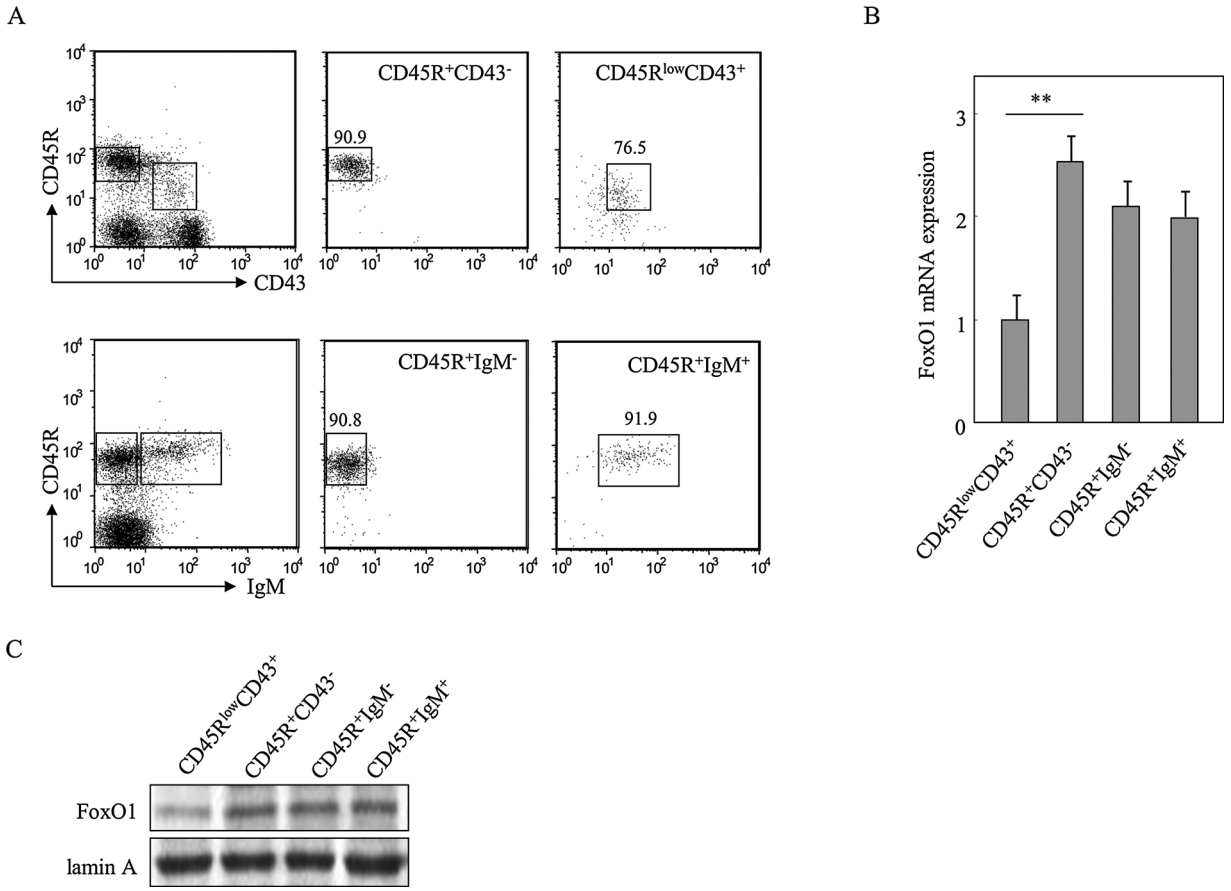

Fig. 1. FoxO1 mRNA expression is increased during the pro-B to pre-B transition. A, FACS analysis and sorting of BM cells from 8-week-old mice. The CD45R$^+$CD43$^-$, CD45R^{low}CD43$^+$, CD45R$^+$IgM- and CD45R$^+$IgM$^+$ cells were gated and sorted. Sorted cell populations, CD45R$^+$CD43$^-$, CD45R^{low}CD43$^+$, CD45R$^+$IgM- and CD45R$^+$IgM$^+$ cells, were routinely reanalyzed and showed 90.9%, 76.5%, 90.8% and 91.9% purity, respectively. B, FoxO1 mRNA expression of sorted CD45R$^+$CD43$^-$, CD45R^{low}CD43$^+$, CD45R$^+$IgM- and CD45R$^+$IgM$^+$ cell fractions by real-time PCR. Data are representative of three experiments. C, FoxO1 protein expression was detected by Western blot. CD45R$^+$CD43$^-$, CD45R^{low}CD43$^+$, CD45R$^+$IgM- and CD45R$^+$IgM$^+$ cells were isolated and lysed. Cell lysate were resolved by 10% SDS-PAGE, and probed with anti-FoxO1 Ab. Expression of lamin A was shown as loading control.

detected for Ebf1, Pax5, VpreB, E2A and NF-kB mRNA levels (Fig. 2D).

To determine the role of FoxO1 in the activation of pre-B cells, we established FoxO1 restored cells, 70Z/3-KD-Re cells. As shown in Fig. 2B, when the exogenous FoxO1 gene was re-introduced into 70Z/3-KD cells, the down-regulation of FoxO1 mRNA expression was recovered. Moreover, Western blot analysis confirmed that FoxO1-siRNA infected 70Z/3-KD cells have dramatically reduced expression of FoxO1 protein, and were restored in 70Z/3-KD-Re cells (Fig. 2C). Consistent with this, the appreciable down-regulation of FoxO1 target proteins, p27 and p21 in the nuclei of 70Z/3-KD cells, and were again restored in 70Z/3-KD-Re cells (Fig. 2C).

Developing B cells rely on a variety of factors that provide signals in conjunction with those from the IL-7Rα. Among the genes we found to be affected by FoxO1 deletion, IL-7Rα showed the most pronounced regulation in the 70Z/3-KD cells (Fig. 2D). To determine whether FoxO1 was required for continuous expression of IL-7Rα on pre-B cells, we therefore examined the levels of IL-7Rα expression in 70Z/3 derivative cells. The expressions of IL-7Rα were significantly down-regulated in 70Z/3-KD cells, and restored in 70Z/3-KD-Re cells in Western blot (Fig. 3A). Flow cytometry analysis also confirmed that the expression of IL-7Rα on the cellular surface were significantly decreased in 70Z/3-KD cells, and recovered in 70Z/3-KD-Re cells (Fig. 3B).

3.3. Loss of FoxO1 impaired the signaling transduction via IL-7Rα

IL-7 is a cytokine essential for lymphocyte differentiation and proliferation [14]. The signaling via IL-7R leads to the growth and survival of B cell progenitors in response to IL-7, which brings Janus Associated Kinase (JAK) in close proximity and allows for its phosphorylation. Phosphorylation of JAKs leads to the recruitment of signal transducer and activator of transcription-5 (STAT5) [14]. We analyzed the changes in the patterns of phosphorylated proteins in 70Z/3 cells after IL-7 stimulation. As expected, the 70Z/3 cells treated with IL-7 for 5, 15 min showed a striking increase in the levels of p-JAK1 compared with the pattern seen in unstimulated cells (Fig. 3C). Furthermore, compared to 70Z/3 cells, the phosphorylation level of JAK1 at 5 min was attenuated by a factor of 3.8 in 70Z/3-KD cells, while the down-regulation of phosphorylation was partly restored in 70Z/3-KD-Re cells. Since the phosphorylations of JAKs leads to the recruitment of STAT protein [15], we next analyzed the changes of STAT5 phosphorylation. The phosphorylation of STAT5 at 15 min was reduced by a factor of 5.3 in 70Z/3-KD cells, whereas those were rescued in the 70Z/3-KD-Re cells (Fig. 3C). JAK1 phosphorylation at 5 min was preceded by the sequential activation of STAT5 at 15 min. These data suggest that FoxO1 regulates early B cell signal transduction by controlling the IL-7Rα expression.

3.4. Knockdown of FoxO1 suppressed pre-B cell proliferation with G0/G1 cell cycle arrest

Given that cell proliferation is another consequence of IL-7Rα signaling, we investigate whether *FoxO1* knockdown influences cell proliferation. The BrdU assay was performed in 70Z/3 derivative cells. In culture, IL-7 selectively enhances pre-B cell survival and proliferation.

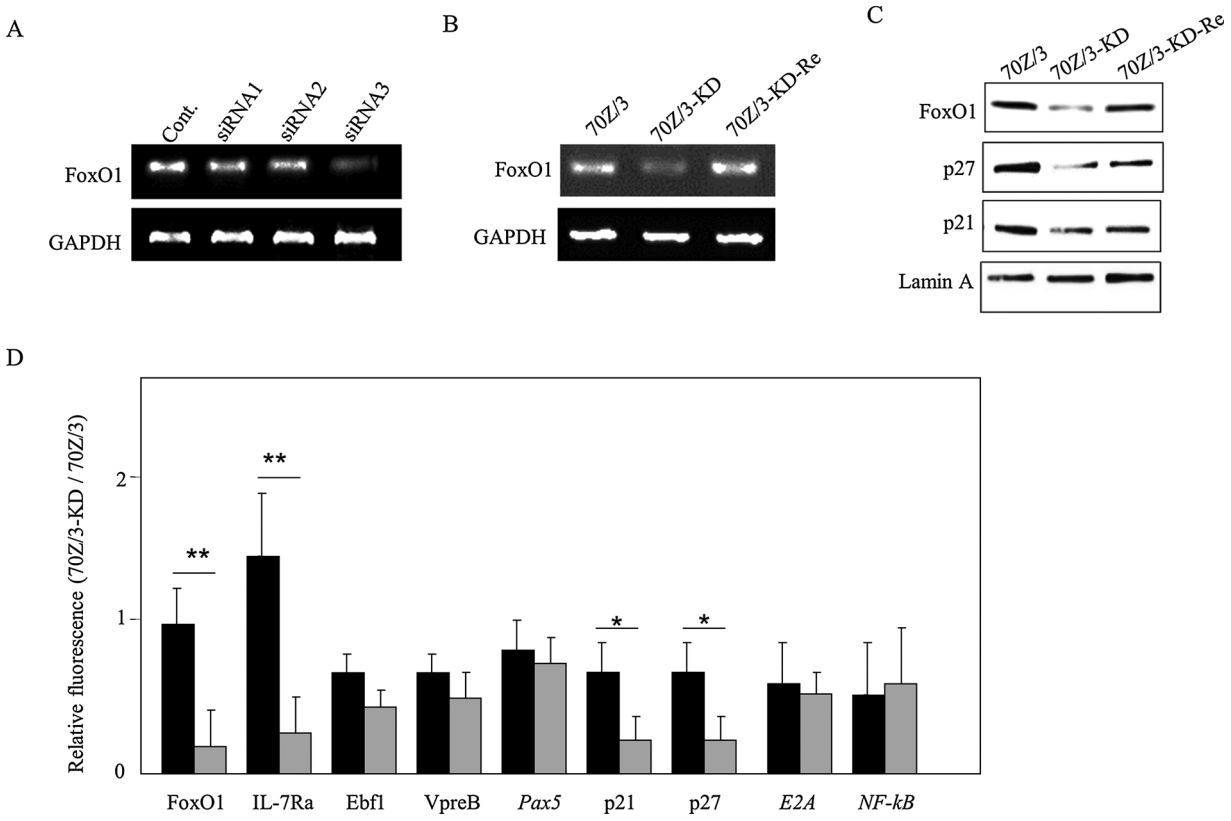

Fig. 2. Establishment of 70Z/3-KD and 70Z/3-KD-Re cells. (A) Expression of FoxO1 mRNA was detected by RT-PCR. Three targeting sequences of FoxO1-siRNA (siRNA1, siRNA2 and siRNA3) and mock sequence were transiently transfected into 70Z/3 cells with *TransIT*-TKO transfection reagent. (B) Expression of FoxO1 mRNA in the 70Z/3, 70Z/3-KD and 70Z/3-KD-Re cells. Recombinant retrovirus particles carrying FoxO1-siRNA3 were infected into 70Z/3 cell, and the G418-resistant clones were selected. 70Z/3-KD-Re cells were established by the transfection of pLHCX-*FoxO1* into 70Z/3-KD cells. (C) Protein expression of FoxO1, p27 and p21 was detected by Western blot. Cell lysate were resolved by SDS-PAGE on a 10% gel, transferred to a PVDF membrane and probed with the anti-FoxO1 Ab, anti-p27 Ab and anti-p21Ab. Expression of lamin A was shown as loading control. (D) Gene-silencing effects of siRNA on the FoxO1 mRNA expression were determined by real-time PCR. Total RNAs were isolated from 70Z/3 and 70Z/3-KD cells. The mRNA expressions of FoxO1, Il7ra, Ebf1, VpreB, Pax5, p21, p27, E2A and NF-kB by real-time PCR analysis. All values were normalized to that of the *GAPDH* gene. Data were representative of the mean ± SD (n = 3) (**, $p < 0.01$).

Therefore the cells were stimulated for 48 h with IL-7. Contrast to the 70Z/3 cells, the proliferation was significantly reduced in the 70Z/3-KD cells. Moreover, the proliferation was significantly increased following the re-introduction of *FoxO1* gene in 70Z/3-KD-Re cells (Fig. 4A). Then, to further address the role of FoxO1 in the pre-B proliferation, fractions containing pre-B cells ($CD45R^+CD43^-$) in the bone marrow were sorted as shown in Fig. 4B, and transfected with special siRNA sequences for the knockdown of *FoxO1* gene. The colony formation of pre-B cells in Complete Methylcellulose Medium in response to IL-7 was distinctively less in *FoxO1* knockdown pre-B cells ($CD45R^+$CD43- + siRNA) (Fig. 4B). To investigate the effect of *FoxO1*gene on cell apoptosis, $CD45R^+$CD43- pre-B and *FoxO1* knockdown pre-B cells were labeled with annexin V and PI, which binds to phosphatidylserine exposed on the cell surface undergoing apoptosis. The percentage of Annexin V^+ PI^+ apoptotic cells was significantly increased by *FoxO1* knockdown (Fig. 4C). These results indicated that FoxO1 affects the cell proliferation by the decreased IL-7Rα response and the increased cell apoptosis.

Several studies reported that FoxO1 could regulate genes involved in cell cycle arrest [16]. We analyzed the cell cycle profile of 70Z/3 derivative cells by flow cytometry. Compared to 70Z/3 cells, 70Z/3-KD cells exhibited an increase in the G_0/G_1 phase proportion and a decrease in the G_2/M phase (Fig. 4D). This result indicated that the cause of the decreased B cell numbers in 70Z/3-KD cells by G_0/G_1 cell cycle arrest.

3.5. *FoxO1 controls IL-7Rα expression in pre-B cells*

IL-7R signaling is required for the survival of early B cells in vivo [17,18]. As FoxO is inactivated with cytokine stimulation and thus could not be directly responsible for the early B cell differentiation, we speculated that loss of FoxO1 could change the expression of IL-7Rα. Ablation of FoxO1 results in a significant down-regulation of IL-7Rα associated with a profound reduction of Il7ra mRNA, but not Il2rg mRNA in 70Z/3-KD cells (Fig. 5A).

Among the genes we observed to be changed by *FoxO1* ablation (Fig. 2D), Il7ra mRNA showed the most significant regulation. Therefore, we sought to clarify whether FoxO1 directly regulates Il7ra expression or not. There are the evolutionarily conserved noncoding regions (ECRs) of *Il7ra* gene upstream of the transcription initiation site [19]. To determine whether FoxO1 directly binds to the *Il7ra* locus, we carried out CHIP experiments using primer sets specific for these ECRs. In isolated pre-B ($CD45R^+CD43^-$) cells, FoxO1 specifically bound to the *Il7ra*-ECR at the region (-3765 to -3575) (Fig. 5B), but not bound to other regions. Our data strongly indicated that FoxO1 is a key upstream regulator of IL-7Rα expression. Moreover, the amount of FoxO1 binding to the *Il7ra*-ECR was detected between 70Z/3, 70Z/3-KD and 70Z/3-KD-Re cells. In the CHIP analysis, FoxO1 bound to *Il7ra*-ECR was markedly reduced in 70Z/3-KD cells, whereas those were restored in the 70Z/3-KD-Re cells (Fig. 5C).

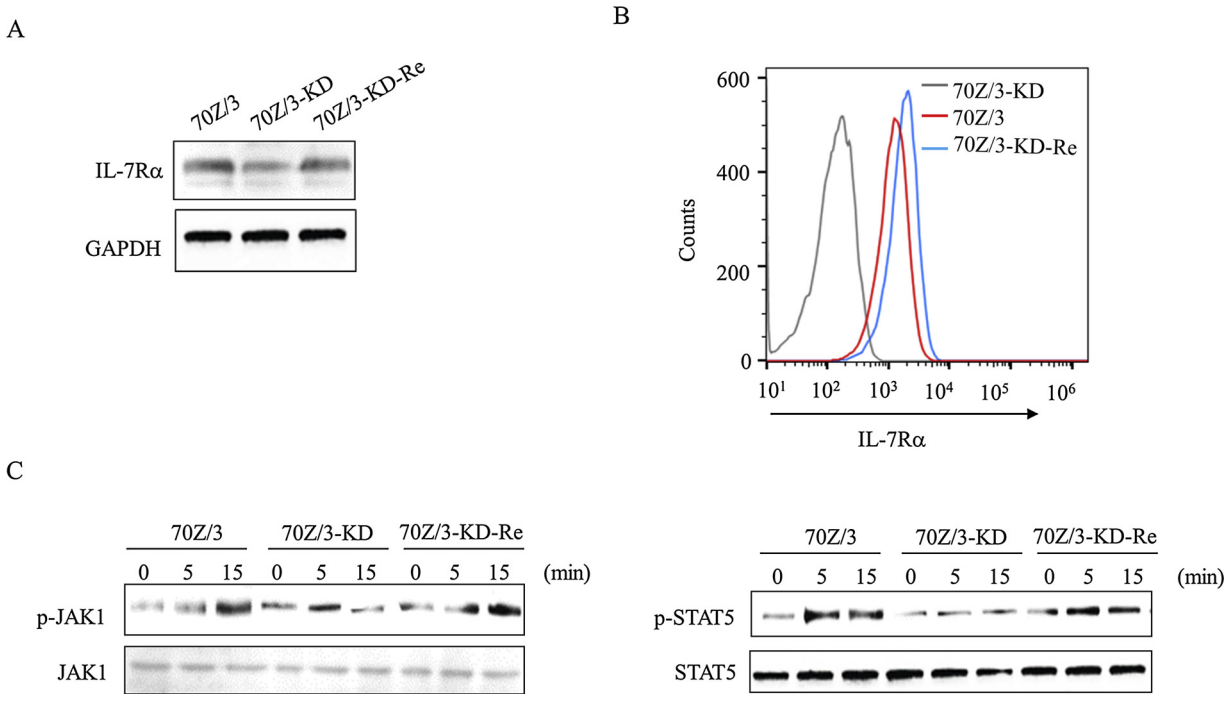

Fig. 3. Down-regulation of phosphorylated JAK1 and STAT5 in 70Z/3-KD cells. A, IL-7Rα expression by Western blot. Cell lysates from 70Z/3, 70Z/3-KD and 70Z/3-KD-Re cells were subjected to 10% SDS-PAGE, transferred to a PVDF membrane, and probed with anti-IL-7Rα Ab. B, FACS analysis of IL-7Rα expression on 70Z/3, 70Z/3-KD and 70Z/3-KD-Re cells. C, Protein expression and phosphorylated forms of JAK1 and STAT5. The serum-starved 70Z/3, 70Z/3-KD, or 70Z/3-KD-re cells were treated with IL-7. Whole cell lysate were resolved by SDS-PAGE on a 7.5% gel, transferred to a PVDF membrane, and probed with the anti-JAK1, anti-p-JAK1, anti-p-STAT5 and anti-STAT5 Abs. The quantitative data of phosphorylated JAK1 were represented as the mean ± SD (n = 3).

4. Discussion

B cell development is tightly controlled by the plural action of transcription factors able to guide B cell commitment, survival, proliferation, Ig rearrangements, receptor editing, and final differentiation into plasma cells [20]. In the present study, we found that the FoxO1 expression was significantly up-regulated at the pre-B cell stage, suggesting that FoxO1 play an important role in the pre-B cell expansion.

While IL-7, SCF, and Flt-3 l all play important roles in enhancing B lymphopoiesis, only IL-7 is absolutely essential, and once a progenitor cell is capable of responding to IL-7, these other factors are no longer required for cell survival and development toward the B lineage. Since IL-7Rα is required for the early B cell proliferation and differentiation, its expression is stringently regulated in developing B cells. Transcription of the IL-7Rα chain is regulated by PU.1 and Flt-3, while FoxO1 is critical in sustaining IL-7Rα expression [10]. As FoxO1 controls IL-7Rα expression and vice versa, up-regulation of FoxO1 by IL-7 constitutes a positive feedback loop supporting the proliferation of pre-B cells. IL-7 withdrawal resulted in FoxO1 recruitment to a region of the *Il7ra* locus previously characterized for glucocorticoid receptor–dependent enhancer activity [21]. At the molecular level, FoxO1 activates transcription of the *Il7ra* gene in pre-B cells [20]. Kerdiles, et al. [22] have shown that FoxO1 binds to ECRs upstream of the Il7ra gene in normal T cells. Consistent with these data, our experiments show that FoxO1 regulates IL-7Rα transcription by binding directly to this ECR in the *Il7ra* locus. Importantly, FoxO1 bound to Il7ra-ECR was markedly reduced in 70Z/3-KD cells and restored in 70Z/3-KD-Re cells by reconstitution of FoxO1 gene. Mechanistically, IL-7Rα signaling is regulated at the level of IL-7Rα expression. As important as the signaling pathways via IL-7Rα, we further investigated how FoxO1 regulates the signal transduction pathways via IL-7Rα and the proliferation. The proliferative response was dramatically reduced following *FoxO1* knockdown by suppressing cells signaling via IL-7Rα, and rescued in the 70Z/3-KD-Re cells. IL-7 or IL-7R knockout mice display a severe block of the early development at pro-B cell stage [17,18]. Dengler, et al. [10] made conditional FoxO1$^{-/-}$ mice with *mb1*^{Cre} and convincingly showed that expression of IL-7Rα was significantly down regulated in the knockout mice. FoxO1 is a critical member of the transcription factor network directing early B cell development. However, deletion of FoxO3 in B cell progenitors does not have a major effect on B cell differentiation [23]. It is conceivable that reduced FoxO1-dependent IL-7Rα expression contribute to impaired cell signaling and early B cell expansion in bone marrow.

Cell cycle progression is a tightly controlled process. To initiate cell growth, mitogens activates cyclin D, cyclin E and CDKs, which are required to trigger DNA synthesis. At the molecular level, FoxO1 could up-regulate the cell-cycle inhibitors p27 and p21, which exhibit important roles during cell cycles, consequently leading to G1/S cell-cycle arrest [24]. In human breast cancer, PKB/Akt pathway phosphorylated p27, caused cytoplasmic p27 accumulation and resistance to TGFβ-mediated G1 arrest [25]. Overexpression of p27Kip1 induced G1 arrest in the presence of IL-7 in an IL-7–dependent T cell line, whereas knockdown of p27Kip1 promoted S phase entry after IL-7 withdrawal [26]. However, we found that the FoxO1 knockdown results in cell cycle G_0/G_1 arrest with p27/p21 down-regulation in pre-B cells. Since IL-7Rα signaling or constitutive STAT5 expression induced the cyclin D2 and cyclin D3 expression, which function to promote cell cycle progression by activating CDKs [27]. Our data indicated that G_0/G_1 arrest in FoxO1 knockdown cells may be associated with attenuated STAT5 signaling, but not with p27/p21 down-regulation. As proper cell cycle regulation seems to be mandatory for cell signaling pathway, it would be interesting to analyze how FoxO1 affect cell cycle regulation.

FoxO1 acts as a key enhancer of IL-7Rα expression in the pre-B cells, and following physiological outcomes of its signaling, such as cell cycle and proliferation. The combined down-regulation of those genes, IL-7Rα, p21 and p27 in FoxO1 knockdown cells is likely to be the most crucial effect resulted from the impaired proliferation of pre-B cells. FoxO1 regulates several other important genes, *Rag1/2* and *Aicda*

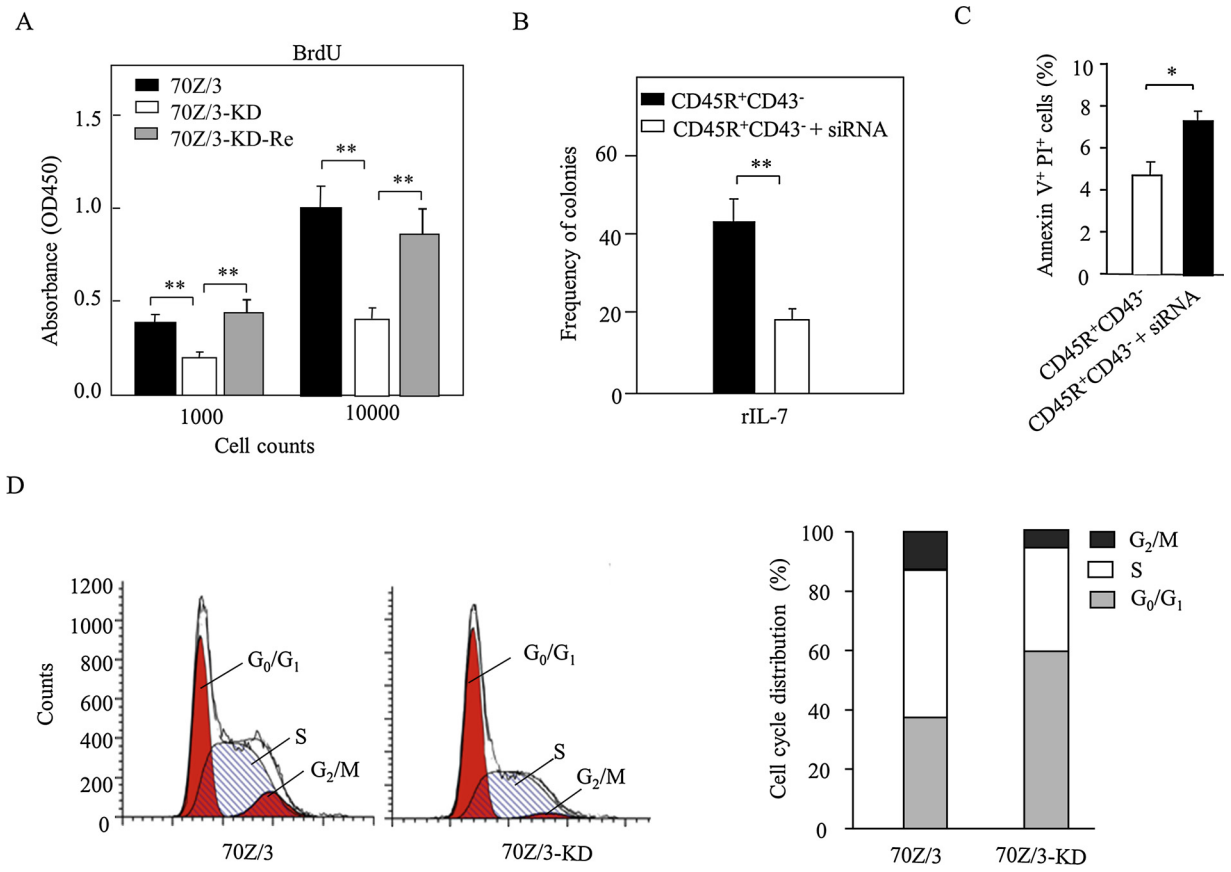

Fig. 4. FoxO1 affects the proliferation and cell-cycle of 70Z/3 cells. A, Effect of FoxO1 on cell proliferation was measured using BrdU assays. Knockdown of FoxO1 significantly reduces the proliferation rate of 70Z/3 cells. Values are expressed as the mean ± SD. **P < 0.01. OD450, optical density at 450 nm. B, Pre-B ($CD45R^+CD43^-$) cell colonies were counted after 5×10^2 cell culture for 7 days in the complete methylcellulose medium in the presence of 10 ng/ml rIL-7. Freshly isolated $CD45R^+CD43$- cells were treated with siRNA specific for FoxO1. Aggregates consisting of over 30 cells were scored as colonies. Data are presented as the mean ± SD of pre-B colony-forming units (n = 3). C, Cell apoptosis. Cells were stained with Annexin-V and PI and assessed by flow cytometry analysis. The percentages of Annexin-V^+ PI^+ cells are displayed. Values are expressed as the mean ± SD. *P < 0.05. D, Cell cycle of the 70Z/3, 70Z/3-KD and 70Z/3-KD-re cells. Stacked columns of cell fractions for each cell-cycle stage (G_0/G_1, S and G_2/M).

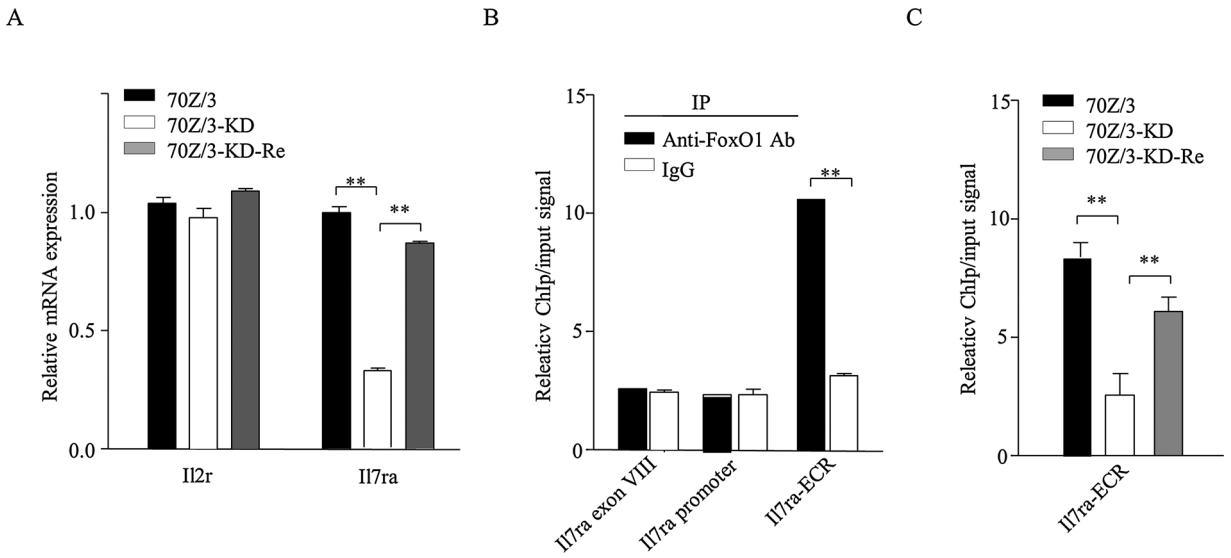

Fig. 5. FoxO1 is required for IL-7Rα expression in pre-B cells and binds to an *Il7ra* enhancer. A, Real time PCR analysis of Il7ra and Il2rg mRNA expression in 70Z/3, 70Z/3-KD and 70Z/3-KD-Re cells. Results are representative of three independent experiments (**, P < 0.001). B. CHIP analysis of FoxO1 binding to the *Il7ra* locus. Freshly isolated $CD45R^+CD43^-$ cells (2×10^6) were fixed with 1% formaldehyde. Results are relative to the value obtained for the control rabbit IgG with anti-FoxO1 Ab. FoxO1-DNA immune complexes were then collected with protein A agarose beads. Results are representative of four independent experiments (**, P < 0.001). C. CHIP analysis of FoxO1 bound to the *Il7ra*-ECR. 70Z/3, 70Z/3-KD and 70Z/3-KD-Re cells were fixed with 1% formaldehyde. FoxO1-DNA immune complexes were then collected with protein A agarose beads.

genes, needed for B cell function [23]. FoxO1 expression is also essential for the downstream activation of Ikaros, Aiolos, and Rag1/2 expression [1,2], thereby future studies will elucidate the role of FoxO1 with other transcriptional factors in regulating early B cell proliferation and differentiation.

5. Conclusion

In the present study, we showed that the FoxO1 expression was significantly up-regulated at the pre-B cell stage, and FoxO1 regulates the expansion of pre-B cells by regulating the expression of IL-7Rα and its signal transduction.

Author contribution

WL and ZL conceived the project, designed and supervised the experiments, NZ, XX and HF performed experiments and the statistical analysis, DZ and WZ carried out animal experiments. All authors reviewed the draft manuscript and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.09.004.

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