Growth of tyrosine kinase inhibitor-resistant Philadelphia-positive acute lymphoblastic leukemia: Role of bone marrow stromal cells

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Abstract. Human bone marrow stromal cells (hBMSCs) may contribute to the growth of tyrosine kinase inhibitor (TKI)-resistant chronic myelogenous leukemia (CML). However, there are certain differences in biology between CML and Philadelphia-positive acute lymphoblastic leukemia (Ph^+ ALL). Little is known about the role and mechanism of hBMSCs on the growth of TKI-resistant Ph^+ ALL. The current study co-cultured hBMSCs with the TKI-resistant SUP-B15. Next, the proliferation of SUP-B15 was detected using a Cell Counting Kit-8. Additionally, quantitative polymerase chain reaction and flow cytometry were used to detect the expression of the associated genes and proteins. The present study explores the role and mechanism of hBMSCs on the growth of TKI-resistant Ph^+ ALL. The current study showed that hBMSCs promoted the proliferation of TKI-resistant Ph^+ ALL. This was shown by the increase in cells in the S+G2-M phase of the cell cycle. It was also found that the expression of cyclins A, C, D1 and E was increased. Apoptosis was inhibited through upregulation of anti-apoptotic genes [B-cell lymphoma-2 (BCL-2) and BCL-extra large] and downregulation of apoptotic genes (BCL-XS, BCL-2-associated X protein, and caspases 3, 7 and 9). Expression of the breakpoint cluster region (BCR)-Abelson murine leukemia viral oncogene homolog 1 (ABL) gene, Wnt5a, and Wnt signaling pathway-associated genes (glycogen synthase kinase-3β, β-catenin, E-cadherin and phosphoinositide 3-kinase) and transcription factors (c-myc, ephrin type-B2, fibroblast growth factor 20 and matrix metalloproteinase 7) was also increased. Furthermore, the expression of drug resistance genes (low-density lipoprotein receptor, multidrug resistance-associated protein and multi-drug resistance gene) was increased and the expression of anti-oncogenes (death-associated protein kinase and interferon regulatory factor-1) was decreased. It was concluded that hBMSCs promote the growth of TKI-resistant Ph^+ ALL by these aforementioned mechanisms. Therefore, targeting hBMSCs may be a promising approach for preventing the growth of TKI-resistant Ph^+ ALL.

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Introduction

In total, 25-30% of adults and 3% of children with Philadelphia-positive acute lymphoblastic leukemia (Ph^+ ALL) have a high rate of complete remission, but also have an extremely poor prognosis due to a high rate of relapse (1). The use of tyrosine kinase inhibitors (TKIs), combined with chemotherapy or subsequent to allogeneic hematopoietic stem cell transplant, improves the outcome of patients with Ph^+ ALL. However, resistance to TKI is a vital aspect of Ph^+ ALL due to the emergence of breakpoint cluster region (BCR)-Abelson murine leukemia viral oncogene homolog 1 (ABL) mutations (2-5). Previous studies have suggested that upon TKI treatment pressure, chronic myelogenous leukemia (CML) stem cells survive due to BCR-ABL kinase-independent mechanisms (6-9).

It is well-known that stromal cells protect CML cells from TKI-induced cell death and mediate the resistance of CML to TKIs (10-15). Human bone marrow stromal cells (hBMSCs) regulate hematopoietic stem cell self-renewal, proliferation and differentiation, which play key roles in the relapse of leukemia (16-18). Targeting hBMSCs may therefore be a promising approach for TKI resistance and the prevention of relapse for Ph^+ ALL, as the leukemia cells of CML are normally dependent on BCR-ABL signaling for growth and survival; solely inhibiting the BCR-ABL tyrosine kinase in Ph^+ ALL is not sufficient to inhibit leukemia cell growth (19,20). Therefore, it is vital to understand the role and mechanism of hBMSCs on the growth of TKI-resistant Ph^+ ALL. However, there are few studies on the role and mechanism of hBMSCs on the growth of TKI-resistant Ph^+ ALL.

Materials and methods

hBMSC culture. hBMSCs were isolated from 20 bone marrow samples obtained between December 2012 and December 2014 and cultured as described by our previous study (21). Bone marrow samples were obtained from newly diagnosed, untreated ALL patients, without infection, aged 18-30 years old. The samples were collected at Xinqiao Hospital, subsequent to obtaining written informed consent from the patients.

Ethical approval was provided by the Ethics Committee of Xinqiao Hospital (Chongqing, China).

Culturing TKI-resistant SUP-B15 cells. The SUP-B15 human Ph+ ALL cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The culture of TKI-resistant SUP-B15 cells (R+SUP-B15) was performed according to our previous study and the literature (20-24). Briefly, when logarithmic growth was observed in the SUP-B15 cells, the culture medium, Iscove's Modified Dulbecco's Medium (IMDM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was changed to medium containing 2 μmol-l Gleevec (Novartis, Basel, Switzerland). The majority of SUP-B15 cells died, and only a small number of SUP-B15 cells survived. For ~3 weeks, the surviving SUP-B15 cells were left to proliferate, and then the associated drug-resistant index [half maximal inhibitory concentration and multi-drug resistance gene (MDR1) level by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)] and BCR-ABL (by RT-qPCR) were detected. These SUP-B15 cells were maintained for the following assays.

Coculture of hBMSCs with SUP-B15 cells. When the passaged hBMSCs reached ~60% confluence, SUP-B15 cells and R+SUP-B15 cells were inoculated for coculture with hBMSCs with 5:1 with IMDM with 10% FBS at 37˚C with 5% CO_2. Subsequent to 1, 4, 7 and 14 days (d1, d4, d7 and d14, respectively) of coculture, SUP-B15 cells and R+SUP-B15 cells were washed twice with PBS prior to subsequent analyses.

Drug sensitivity. The R+SUP-B15 cells were collected and were continuously cultured with hBMSCs with IMDM medium supplemented with 10% FBS and containing 250 ng-ml cyclophosphamide (Cy; Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) for 1, 4, 7 and 14 days. The surviving cells were washed twice with PBS prior to subsequent analyses.

Clone forming test. A 2-layer soft agar culture system was used for this assay. A total of $1x10^3$ cocultured R+SUP-B15 cells per ml were plated in a volume of 4 ml IMDM (0.7% agar) over 3-ml base layers (1.2% agar) in 60-mm Petri dishes. The cultures were incubated in humidified 37˚C incubators with an atmosphere of 5% CO_2, and control plates were monitored for growth using an inverted microscope on d1, d4, d7 and d14. Colony counts were obtained using an inverted microscope from at least 5 fields of view at a magnification of x100. Objects presenting a circular profile in 2 dimensions with a minimum diameter of 60 μm were considered to be colonies.

Cell Counting Kit-8 (CCK-8) assay. The cocultured R+SUP-B15 cells and SUP-B15 cells ($1x10^4$-ml; 200 μl-well) were seeded into 96-well plates as aforementioned and cultured for 4 h. The single culture R+SUP-B15 cells ($1x10^4$-ml; 200 μl-well) were plated as a control group. The proliferation was assessed according to our previous study using CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) (25).

Cell cycle distribution. The R+SUP-B15 cells cultured with hBMSCs and R+SUP-B15 cells cultured with hBMSCs combined with Cy were collected and stained with propidium iodide (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). The nuclei were analyzed using flow cytometry (FACSCalibur; Beckon Dickinson, Franklin Lakes, NJ, USA). The percentage of cells at each phase of the cell cycle was estimated from the DNA-content histograms.

Flow cytometry for cyclins. The cocultured R+SUP-B15 cells were collected and stained for cyclins A (catalog no., C4710), D1 (catalog no., C7464), E (catalog no., SAB4503514) and C (catalog no., SAB4504700) (Sigma-Aldrich; Merck Millipore) at 4˚C overnight. The cells were washed with 0.01 mol-l PBS and stained with fluorescein isothiocyanate-anti-IgG (Sigma-Aldrich; Merck Millipore) at 37˚C for 2 h. The positive staining was detected using flow cytometry (FACSCalibur; Beckon Dickinson).

Flow cytometry for apoptosis. The cocultured cells and cocultured cells combined with Cy were collected. An Annexin V binding and propidium iodide uptake assay (R&D Systems, Minneapolis, MN, USA) was employed for apoptosis analysis, as previously described (26).

Tumor forming test. Male NOD-SCID mice (n=20) were obtained from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). All mice were bred and maintained under specific pathogen-free conditions at 25˚C in a 12 h light-dark cycle, with 50% humidity, in the Animal Center of the Third Military Medical University (Chongqing, China). The Ethical Review Committee of the Third Military Medical University (Chongqing, China) approved the experimental protocol. The SUP-B15 cells and R+SUP-B15 cells cultured with hBMSCs for 1, 4, 7 and 14 days were washed twice with serum-free RPMI-1640 medium. These cells were resuspended at a density of $1x10^7$ cells-ml in serum-free RPMI-1640 medium. The mice were anesthetized with ether, and the cells were inoculated subcutaneously into SCID mice by a dorsal injection at a dose of $5x10^6$ cells per mouse. Mice with tumors 1.5-2.0 cm in diameter were sacrificed during the 2-3-week follow-up period subsequent to inoculation with different coculture cells. The subcutaneous tumor was excised from the mice. The length, width and height of the tumor were measured using the Somers scale (27). Tumor tissues were sliced at 4-μm thickness, and Harris hematoxylin and eosin staining (American MasterTech, Lodi, CA, USA) was performed.

RT-qPCR. Subsequent to 1, 4, 7, and 14 days of coculture, R+SUP-B15 cells were collected. Total RNA was extracted from the cells using TRIzol reagent and was then reverse-transcribed into cDNA. The mRNA expression of cyclins A, D1, E and C, caspases 3, 7, 8 and 9, the BCR-ABL gene, Wnt signaling pathway-associated genes [c-myc, matrix metalloproteinase 7 (MMP7), ephrin type-B2 (EphB2) and fibroblast growth factor 20 (FGF20)], anti-apoptotic genes [B cell lymphoma (Bcl)-2 and Bcl-extra large (XL)], apoptotic genes [Bcl-short isoform (xs) and Bcl-associated X protein (Bax)], anti-oncogenes [interferon regulatory factor (IRF-1) and death-associated protein kinase (DAPK)] and drug resistance genes [low-density lipoprotein receptor (LRP),

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Sense, 5'-3'	Antisense, 5'-3'
Cyclin A	TGTCACCGTTCCTCCTTG	GCATCTTCACGCTCTATTT
Cyclin D1	AGGAACAGAAGTGCGAGGAG	AGGCGGTAGTAGGACAGGAAG
Cyclin C	TGATTGCTGCTGCTACTT	CCATTTGGACCCTGCTCT
Cyclin E	CTGGATGTTGACTGCCTTGA	CCGCTGCTCTGCTTCTTAC
c-myc	GGGCTTTATCTAACTCGCTGTA	TATGGGCAAAGTTTCGTG
MMP7	TATGGGACATTCCTCTGA	TCTGCCTGAAGTTTCTATT
EphB2	AGGACGATACCTCAGACCC	AGGACATCACCTCCCACAT
FGF20	CATCTTTAGGGAGCAGTTT	TGGAGTTCCGTCTTTGTTA
BCR/ABL	CTACGAGGACGCCGAGTTGA	TGCGAGTTCTGCGAGGGA
Bcl-2	ATGGCAAATGACCAGCAGA	GCAGGATAGCAGCACAGGA
Bcl-XL	GGAGGCAGGCGACGAGTTT	GGACGGAGGATGTGGTGGA
Bcl-Xs	TGAGGGAGGCAGGCGACGAGTT	ATGGCGGCTGGACGGAGGAT
Bax	TTTGCTTCAGGGTTTCATCCA	GAGACACTCGCTCAGCTTCTTG
Caspase-3	TTTGGTACTGTATTTCCCTC	GCAGTTAAGTCATCCGTGT
Caspase-7	AGAATGTGGGTATTGAGTGT	GAGGGCAGGATAGGTGAGA
Caspase-8	GCTGGTGGCAATAAATAC	AGAAGGCATAAAGCAAGT
Caspase-9	CGAACTAACAGGCAAGCA	TCACCAAATCCTCCAGAAC
IRF1	CTGAGTCTACAGCGTGTCTTC	CTGGGATTGGTGTTATGC
DAPK	TTCGGCTCAAATCCCAATA	CCATCTGACACCGTCTTACA
MDR1	AAGCCTACAGCCGTGTCC	TCCTCCTCTTGGGTTTGG
MRP	CGCTGAGTTCCTGCGTAC	GTTCTGCGGTGCTGTTGT
LRP	CTTCAAGAACGCAGTGGTG	CAGTAGAGTTTGCTTTCAGGGA
β-actin	GTGGACATCCGCAAAGAC	AAAGGGTGTAACGCAACTAA

MMP7, matrix metalloproteinase 7; EphB2, ephrin type-B2; FGF20, fibroblast growth factor 20; BCR/ABL, breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1; Bcl-2, B cell lymphoma-2; Bcl-XL, Bcl-extra large; Bax, Bcl-associated X protein; DAPK, death-associated protein kinase; IRF-1, interferon regulatory factor-1; MDR1, multi-drug resistance gene; MRP, multidrug resistance-associated protein; LRP, low-density lipoprotein receptor.

multidrug resistance-associated protein (MRP) and MDR1] were analyzed by qPCR using β-actin as an internal standard. The fold change of target gene expression in the experimental group compared with the control group was calculated using $2^{-\Delta\Delta Cq}$, according to a previous study (19). The primers used were shown in Table I. The amplification conditions were as follows: 95˚C for 3 min; 40 cycles of 95˚C for 20 sec, 56˚C for 20 sec and 72˚C for 20 sec; 95˚C for 15 sec; 60˚C for 15 sec; 60-95˚C for 20 min; and 95˚C for 15 sec.

Western blot analysis. Subsequent to 1, 4, 7, and 14 days of coculture, R+SUP-B15 cells were collected. The expression of cyclins A, D1, E and C, BCR-ABL, Wnt signaling pathway-associated proteins (c-myc, MMP7, EphB2 and FGF20), anti-apoptotic proteins (Bcl-2 and Bcl-XL), apoptotic proteins (Bcl-xs and Bax), caspases 3, 7, 8 and 9 and drug resistance proteins (MDR1, MRP and LRP) was analyzed by western blot analysis, according to the manufacturer's protocol. Briefly, R+SUP-B15 cells from different time points were washed and resuspended in ice-cold PBS at a concentration of $1x10^6$ cells-ml and were incubated in ice-cold RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China). The cell suspensions were centrifuged at 10,000 x *g* at 4˚C and the supernatant was collected after 20 min. The Bradford assay (Beyotime Institute of Biotechnology) was used to measure the supernatant protein content. A total of 50 mg protein extracts were separated by SDS-PAGE with 10% polyacrylamide gels and were subsequently transferred onto nitrocellulose membranes after thawing and boiling the protein samples in Laemmli buffer (Sigma-Aldrich; Merck Millipore) for 5 min. Subsequent to blocking the membranes with 5% non-fat milk, the membranes were incubated overnight at 4˚C with antibodies against: Cyclin A (catalog no., ab87359; dilution, 1:1,000; Abcam, Cambridge, UK); cyclin D1 (catalog no., ab134175; dilution, 1:10,000; Abcam); cyclin E (catalog no., ab33911; dilution, 1:2,000; Abcam); cyclin C (catalog no., ab78868; dilution, 1:500; Abcam); BCR-ABL (catalog no., ab187831; dilution, 1:500; Abcam); c-myc (catalog no., BM0238; dilution, 1:400; Shanghai Qiancheng Biological Technology Co., Ltd., Shanghai, China); MMP7 (catalog no., ab38996; dilution, 1:1,000; Abcam); EphB2 (catalog no., ab5418, 1:500; Abcam); FGF20 (catalog no., ab139054, 1:500, Abcam); BCL-2 (catalog no., ab32124; dilution, 1:1,000; Abcam); BCL-XL (catalog no., 10455-R016-50; dilution, 1:5,000; Sino Biological, Inc., Beijing, China); BCL-XS (catalog no., 101579-T32-50; dilution, 1:5,000; Sino Biological, Inc.); Bax (catalog no., ab32503; dilution, 1:2,000; Abcam); caspase 3 (catalog no., ab90437; dilution, 1:1,000; Abcam); caspase 7 (catalog no., ab201959;

dilution, 1:1,000; Abcam); caspase 8 (catalog no., ab32397; dilution, 1:1,000; Abcam); caspase 9 (catalog no., ab202068; dilution, 1:1,000; Abcam); MDR1 (catalog no., ab170904; dilution, 1:1,000; Abcam); MRP (catalog no., ab32574; dilution, 1:500; Abcam); LRP (catalog no., ab32574; dilution, 1:500; Abcam); and β-actin (catalog no., ab8226; dilution, 1:5,000; Abcam). The cells were then incubated with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated (catalog no., ab6721; dilution, 1:5,000; Abcam) and goat anti-mouse IgG HRP-conjugated (catalog no., ab6789; dilution, 1:10,000; Abcam) secondary antibodies at room temperature for 1 h after washing the membranes. The membranes were then developed with enhanced chemiluminescence solution (Thermo Fisher Scientific, Inc.). β-actin was used as a control.

ELISA assay. The supernatant of cocultured cells was collected. The expression of Wnt5a was detected by ELISA kit from Yi Han Biological Technology Co., Ltd., (Shanghai, China) according to the manufacturer's protocol.

Laser confocal microscopy. R+SUP-B15 cells cocultured with hBMSCs were collected and washed twice with PBS. The cells were permeated with 0.1% Triton X-100 for 15 min, then washed with PBS and neutralized with 4% bovine serum albumin (Roche Diagnostics, Basel, Switzerland) for 30 min. Antibodies against E-cadherin (rabbit monoclonal antibody; catalog no., SAB5500022), β-catenin (mouse monoclonal antibody; catalog no., C4231), phosphoinositide 3-kinase (PI3K; rabbit monoclonal antibody; catalog no., SAB5500162) and glycogen synthase kinase (GSK)-3β (rat polyclonal antibody; catalog no., G7914) (Sigma-Aldrich; Merck Millipore) were added overnight at 4˚C, at a dilution of 1:200 and then the cells were washed with PBS and FITC-anti-IgG was added for 1 h at 37˚C. The cells were washed with PBS and images were captured.

Scanning electron microscopy. Single culture R+SUP-B15 cells and R+SUP-B15 cells cocultured with hBMSCs were collected. The cells were washed with 0.01 phosphate buffer saline (PBS) (catalog no., PBS1; Sigma-Aldrich; Merck Millipore) subsequent to centrifugation at 1000 x *g* for 5 min for double times, then the cells were dried onto polylysine-coated glass slides at room temperature for ~1 h. Subsequent to being washed with 0.01 PBS, the slides was fixed with 5% lutaric dialdehyde at 4˚C for 1 h, then washed twice with 0.01 PBS for >30 min each time. Sequential fixation was performed with 1% osmic acid at 4˚C for 1 h. Subsequent to washing, the slides were dehydrated in turn by ethanol (catalog no., PHR1373; Sigma-Aldrich; Merck Millipore) with the concentrations of 30, 50, 70, 80, 90 and 100%. Replacement with liquid CO_2 was performed by critical point drying, and the CO_2 was removed while in a supercritical state. The dry specimen was sputter-coated with gold palladium alloy (catalog no., GF48929967; Sigma-Aldrich; Merck Millipore). The morphology of the cells was then observed using a scanning electron microscope.

Statistical analysis. Statistical analysis was performed using the SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA). The Wilcoxon signed-rank test was used to determine differences between paired samples, and the Mann-Whitney U test was used to determine differences between different groups. Tukey's test was used to control for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference. Average values were expressed as the mean ± standard error of the mean.

Results

Cell proliferation

Proliferation of R+SUP-B15 cells. All cells demonstrated an increase in proliferation as culture time increased, and the peak proliferation rate was reached on d4 for R+SUP-B15 cells. However, the peak proliferation rate was achieved on d7 for R+SUP-B15 cells cultured with hBMSCs. The proliferation of R+SUP-B15 cells cultured with hBMSCs was faster than R+SUP-B15 cells cultured alone on d7 and d14 ($P<0.05$; Fig. 1A).

Clone formation. The clones for R+SUP-B15 cells cultured with hBMSCs were observed on d4. However, the clones for SUP-B15 cells cultured with hBMSCs were observed on d7. All numbers of clones increased with increasing culture time. The number of clones was increased in the R+SUP-B15 cells compared with the SUP-B15 cells cultured with hBMSCs on d14 ($P=0.021$; Fig. 1B).

Cell cycle analysis and cyclin expression. The rate of S+G2-M phase gradually increased with increasing culture time, and the difference was statistically significant between d1 and d7 ($P<0.05$; Fig. 1C). The expression of cyclins was also detected using flow cytometry. The results showed that the expression of cyclins A, C, D1 and E gradually increased with increasing culture time, and the difference was statistically significant between d1 and d7 ($P<0.05$; Fig. 1D). Western blot analysis revealed that cyclins A, C, D1 and E gradually increased with increasing culture time, and the difference was statistically significant on d4 ($P<0.05$; Fig. 1E). The RNA expression of cyclins A, C, D1 and E also gradually increased with increasing culture time, and the difference was statistically significant on d4 for cyclin A and E and on d7 for cyclin C and D1 ($P<0.05$; Fig. 1F).

Tumor formation in NOD-SCID mice. The tumors were observed in the skin of nude mice. The largest tumor was observed from the cells cultured with hBMSCs for 14 days. There was no difference in the size and weight of the tumors between the cell types prior to d7 ($P>0.05$). The size and weight was increased in SUP-B15 cells compared to R+SUP-B15 cells cultured for 14 days ($P<0.05$). Numerous tumor cells were observed in the tumor with Harris hematoxylin staining, and neovascularization was observed in the cells cultured for 14 days (Fig. 1G).

Expression of BCR-ABL and Wnt signaling pathway-associated genes

Wnt gene expression. The expression of Wnt5a gradually increased with increasing culture time. The expression was significantly higher in R+SUP-B15 cells compared with SUP-B15 cells on d4, d7 and d14 ($P<0.05$) (Fig. 2A).

Expression of Wnt signaling pathway-associated proteins. Laser confocal microscopy was used to detect the expression of Wnt signaling pathway-associated proteins. The expression of PI3K, GSK-3β, E-cadherin and β-catenin gradually increased with increasing culture time.

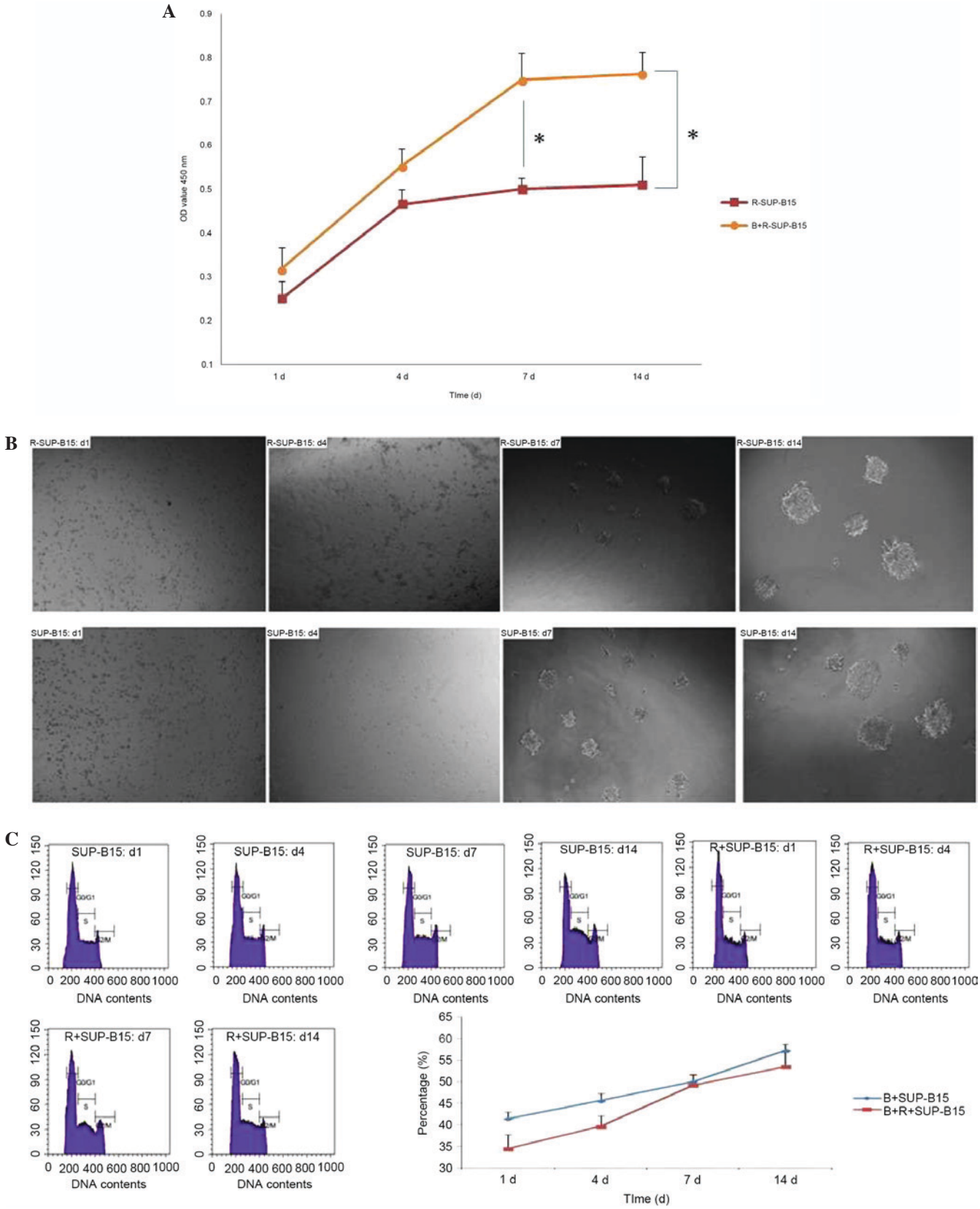

Figure 1. Proliferation of R+SUP-B15 cells cultured with hBMSCs. hBMSCs promoted the proliferation of R+SUP-B15 cells and gradually increased during culture. (A) Proliferation curves of the two cell lines. (B) Clone-forming assay (magnification, x100). (C) Cell cycle analysis. *P<0.05. R+SUP-B15, tyrosine kinase inhibitor-resistant SUP-B15 cells; OD, optical density; hBMSCs, human bone marrow stromal cells; d, days.

Green fluorescence protein was mainly located in the cell membranes and cytoplasm, and no change in location was observed (Fig. 2B).

Transcription factors. hBMSCs promoted the protein expression of c-myc, EphB2, MMP7 and FGF20 and increased with increasing culture time, and the difference was statistically

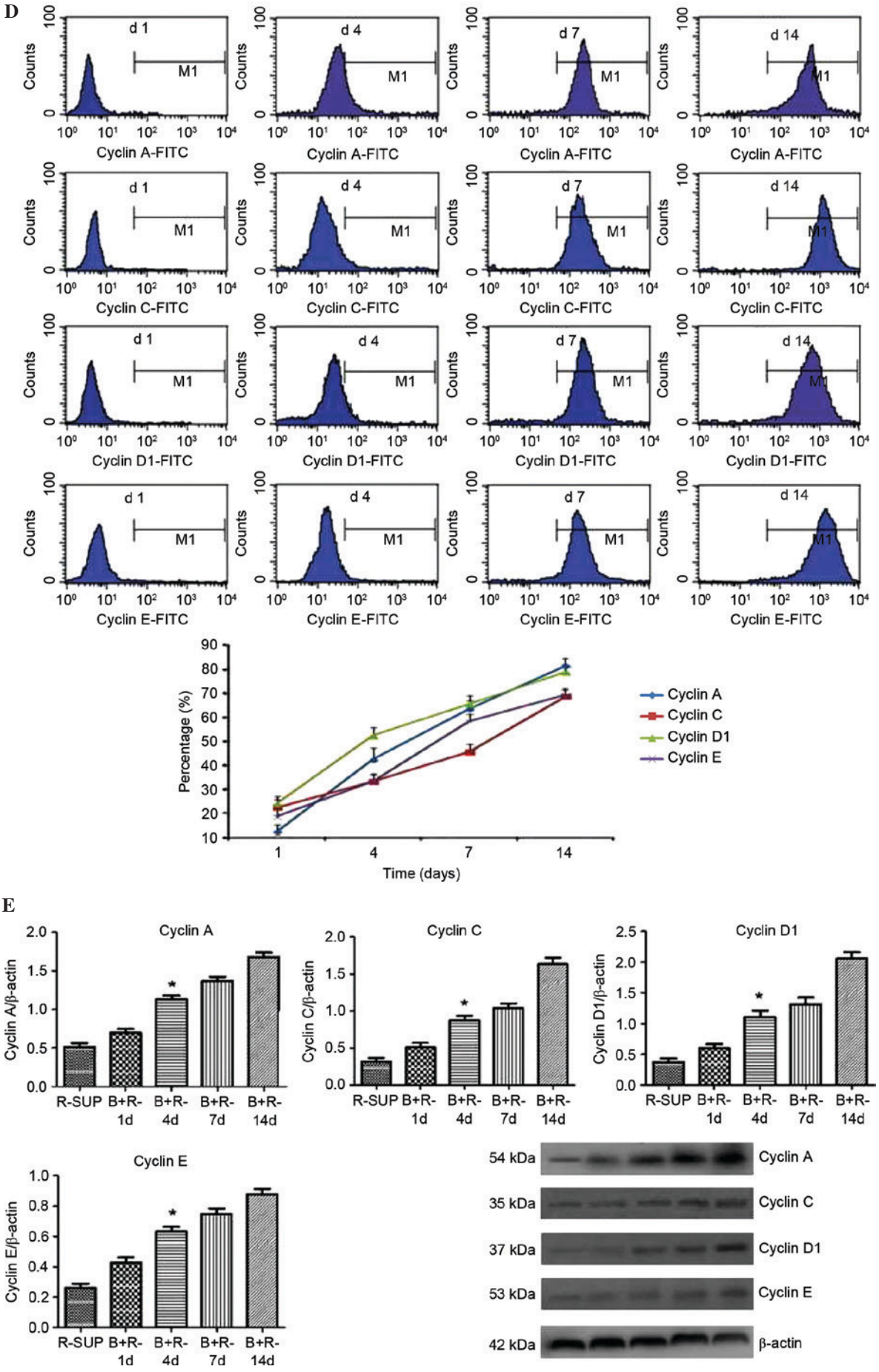

Figure 1. Continued. (D) Expression of cyclins detected by flow cytometry. (E) Expression of cyclin proteins detected by western blot analysis. *P<0.05. FITC, fluorescein isothiocyanate; d, days.

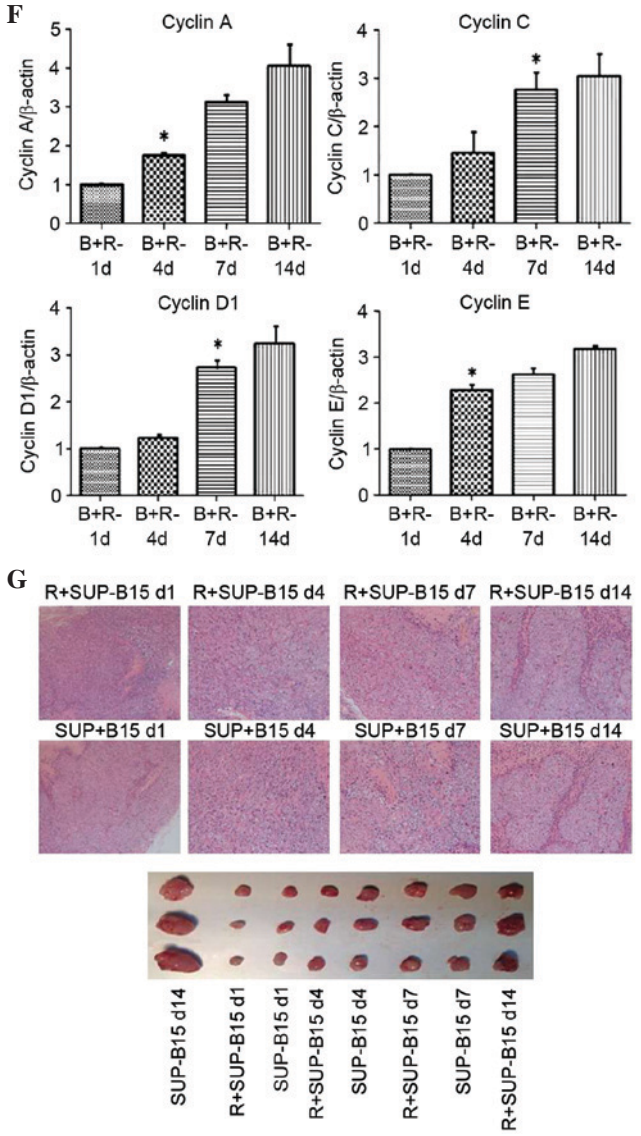

Figure 1. Continued. (F) Expression of cyclin mRNA detected by quantitative polymerase chain reaction. (G) Tumor formation in nude mice *P<0.05. R+SUP-B15 cells, tyrosine kinase inhibitor-resistant SUP-B15 cells; d, day.

significant on d4 compared with d1 (P<0.05; Fig. 2C). mRNA expression of c-myc, EphB2, MMP7 and FGF20 was detected by qPCR, and the results showed that hBMSCs promoted the mRNA expression of c-myc, EphB2, MMP7 and FGF20 and increased with increasing culture time. The difference was statistically significant on d7 for EphB2 expression and on d14 for FGF20, c-myc and MMP7 expression (P<0.05; Fig. 2D).

BCR-ABL gene. Western blotting showed that hBMSCs promoted the expression of BCR-ABL and gradually increased with increasing culture time, and the difference was statistically significant on d4 compared with d1 (P<0.05; Fig. 2E). The mRNA expression of BCR-ABL detected with qPCR was also promoted by hBMSCs and gradually increased with increasing culture time, and the difference was statistically significant on d7 compared with d1 (P<0.05; Fig. 2F).

Cell apoptosis

Hoechst 33342-PI staining. Fluorescence staining with Hoechst 33342 gradually decreased with increasing culture time, and a significant decrease was observed on d14 compared with d1. However, no change was observed for the fluorescence staining with PI at any time point (data not shown).

Expression of anti-apoptotic and apoptosis-associated gene mRNA. mRNA expression of anti-apoptotic and apoptosis-associated genes was detected using qPCR, and the results showed that hBMSCs promoted the expression of anti-apoptotic genes (BCL-2 and BCL-XL) and decreased the expression of apoptotic genes (BCL-XS, Bax and caspases 3, 7 and 9) during the culture period. The difference was statistically significant on d4 for BCL-2, BCL-XL, Bax and caspase 9, and on d7 for BCL-XS, caspase 3 and caspase 7 (P<0.05). No change was observed for caspase 8 (Fig. 3B).

Expression of anti-apoptotic and apoptosis-associated proteins. The protein expression of anti-apoptotic and apoptosis-associated genes was further detected with western blot analysis, and the results showed that hBMSCs promoted the expression of anti-apoptotic proteins (BCL-2 and BCL-XL) and decreased the expression of apoptotic proteins (BCL-XS, Bax and caspases 3, 7 and 9) over the culture period. The difference was statistically significant on d4 compared with d1 (P<0.05). No change was observed for caspase 8 (Fig. 3C).

Morphological changes. The nucleus of R+SUP-B15 cells cultured with hBMSCs became smaller over time during the culture period while the cytoplasm, organelles, granucles and Golgi complexes became numerous, and the chromatin became slimmer. At the end of culture, the cells grew vigorously (Fig. 3D).

Drug sensitivity

Drug resistance genes. The expression of the drug resistance genes LRP, MRP and MDR1 was increased in R+SUP-B15 cells cultured with hBMSCs compared with R+SUP-B15 cells cultured alone and increased with increasing culture time. The difference between the two culture groups was statistically significant from d4 compared with d1 (P<0.05; Fig. 4A). qPCR was also used to detect mRNA expression. mRNA expression of LRP, MRP and MDR1 was increased in R+SUP-B15 cells cultured with hBMSCs compared with R+SUP-B15 cells cultured alone and increased with increasing culture time. The difference was statistically significant from d4 compared with d1 (P<0.05; Fig. 4B).

Anti-oncogene expression. The expression of DAPK and IRF-1 was increased in R+SUP-B15 cells cultured with hBMSCs compared with R+SUP-B15 cells cultured alone and decreased with increasing culture time, and the difference was statistically significant on d4 compared with d1 (P<0.05; Fig. 4C).

Cell cycle progression. The proportion of cells in the S+G2-M phase gradually increased with increasing culture time in R+SUP-B15 cells cultured with hBMSCs combined with Cy, and the difference was statistically significant from d7 compared with d1 (P<0.05; Fig. 4D).

Cell apoptosis. No change was observed in the rate of apoptosis for R+SUP-B15 cells cultured with hBMSCs combined with Cy (P>0.05; Fig. 4E).

Discussion

The tumor microenvironment not only plays a pivotal role during cancer progression and metastasis, but also has

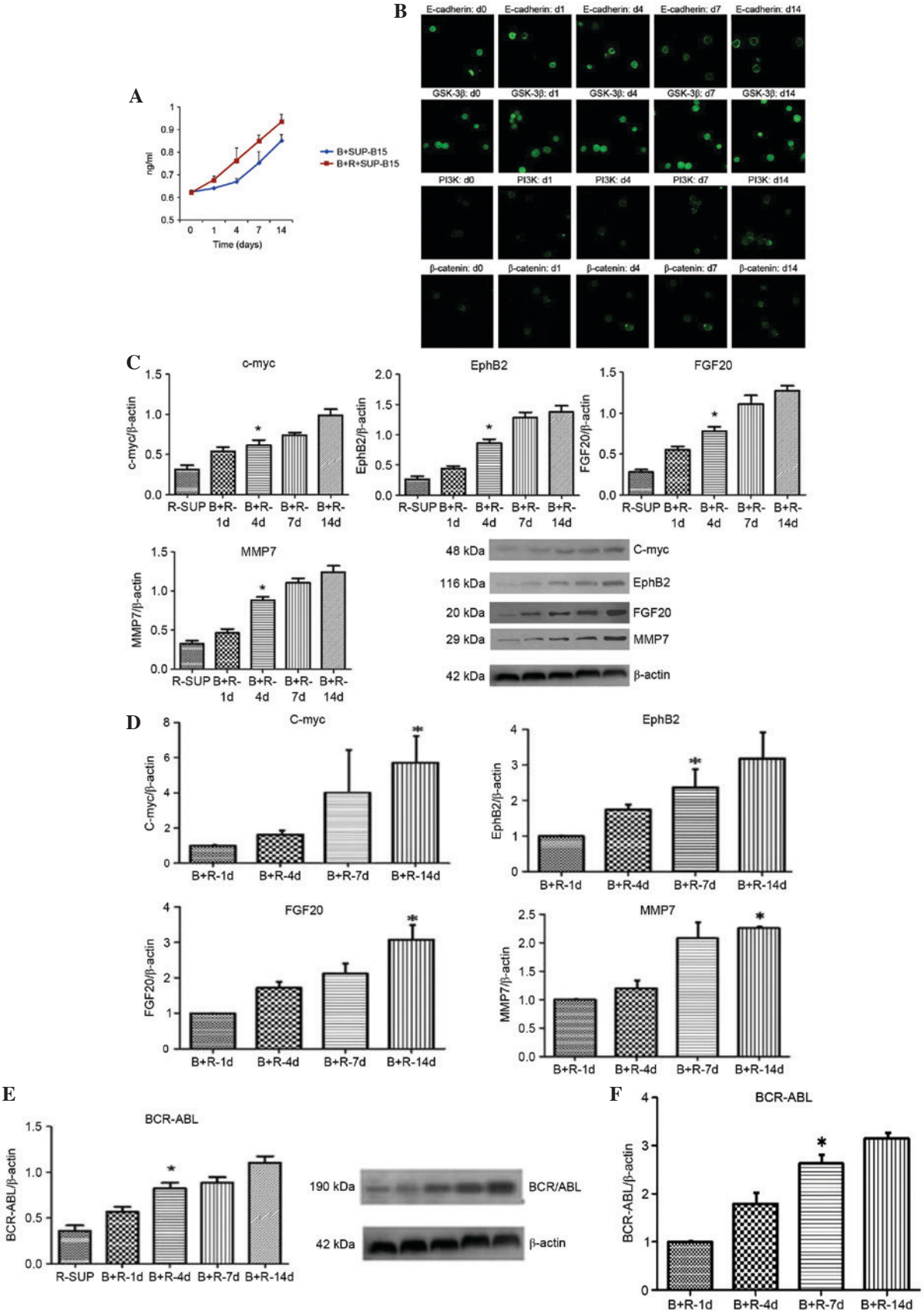

Figure 2. The expression of BCR/ABL and Wnt signaling pathway-associated genes and transcription factors in R+SUP-B15 cells cultured with hBMSCs. hBMSCs promoted the expression of BCR/ABL and Wnt signaling pathway-associated genes and transcription factors and gradually increased during culture. (A) Expression of Wnt. (B) Expression of Wnt signaling pathway-associated genes (PI3K, GSK-3β, E-cadherin and β-catenin) (magnifciation, x1,000). (C) Protein expression of Wnt signaling pathway-associated transcription factors (c-myc, EphB2, FGF20 and MMP7). (D) mRNA expression of Wnt signaling pathway-associated transcription factors (c-myc, EphB2, FGF20 and MMP7). (E) Protein expression of BCR/ABL. (F) mRNA expression of BCR/ABL. *P<0.05. PI3K, phosphoinositide 3-kinase; GSK-3β, glycogen synthase kinase-3β; BCR/ABL, breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1; EphB2, ephrin type-B2; FGF20, fibroblast growth factor 20; MMP7, matrix metalloproteinase 7; d, days; R+SUP-B15 cells, tyrosine kinase inhibitor-resistant SUP-B15 cells; hBMSCs, human bone marrow stromal cells.

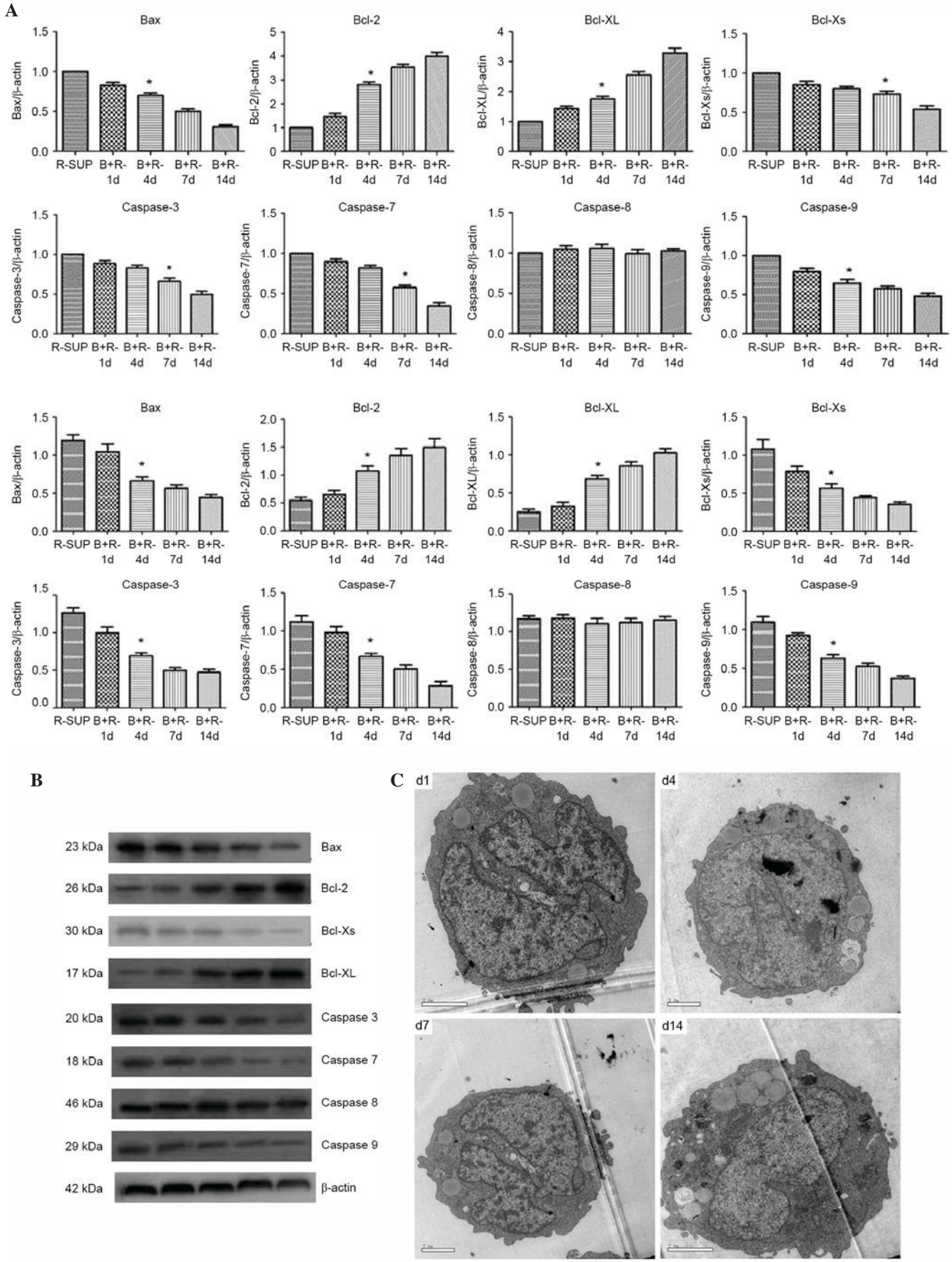

Figure 3. Apoptosis of R+SUP-B15 cells cultured with hBMSCs. hBMSCs inhibited the apoptosis of R+SUP-B15 cells and gradually increased during culture. (A) mRNA expression of anti-apoptotic genes (BCL-2 and BCL-XL), apoptotic genes (BCL-XS and Bax) and caspases 3, 7, 8 and 9. (B) Protein expression of anti-apoptotic genes (BCL-2 and BCL-XL), apoptotic genes (BCL-XS and BAX) and caspases 3, 7, 8 and 9. (C) Morphological changes of R+SUP-B15 cells. Scale bar, 2 μm. *P<0.05. Bcl-2, B cell lymphoma-2; Bax, Bcl-associated X protein; Bcl-XL, Bcl-extra large; d, day; R+SUP-B15 cells, tyrosine kinase inhibitor-resistant SUP-B15 cells; hBMSCs, human bone marrow stromal cells; PI, propidium iodide.

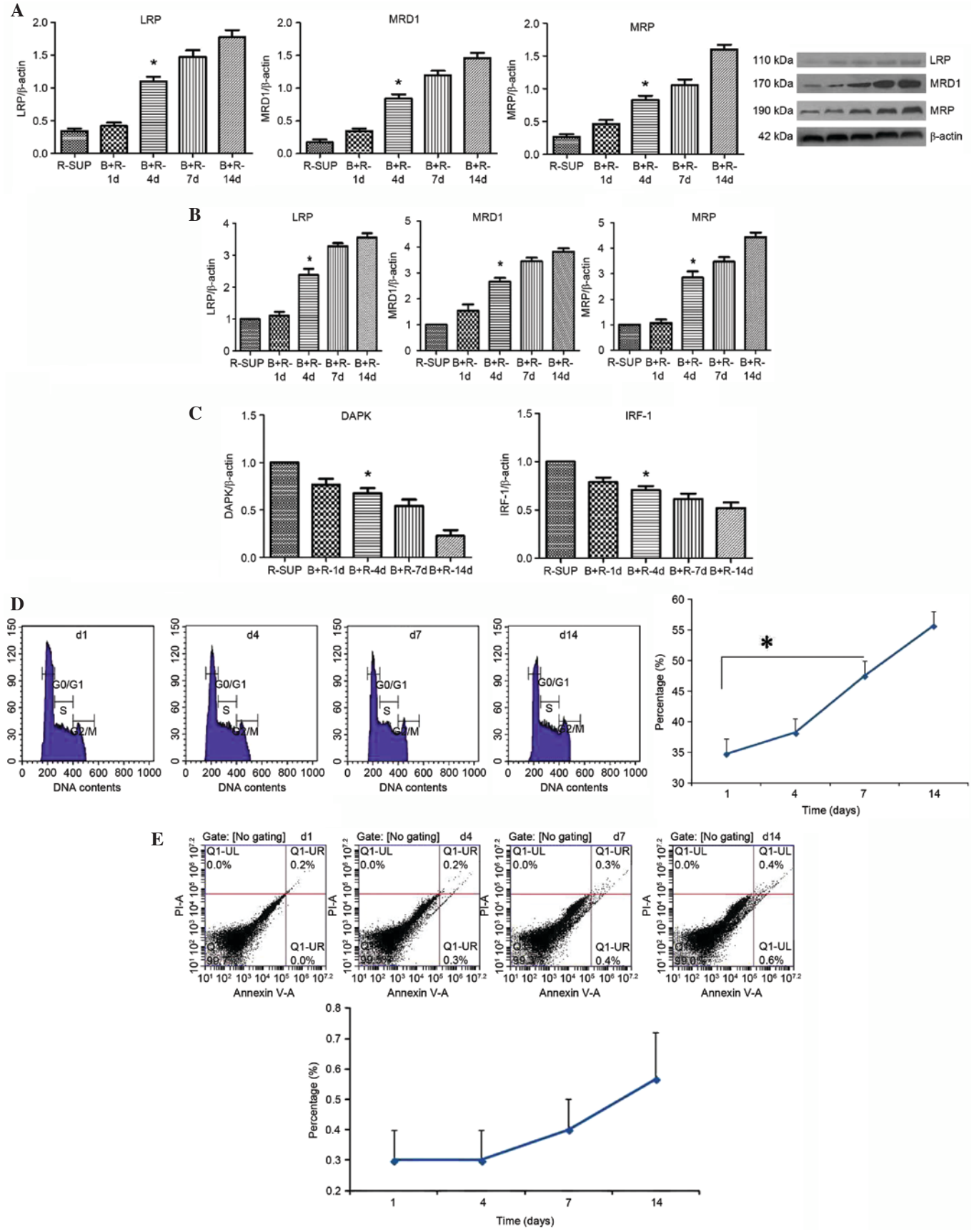

Figure 4. Drug sensitivity of R+SUP-B15 cells cultured with hBMSCs. hBMSCs inhibited anti-oncogene expression, promoted drug resistance gene expression and gradually increased during culture. No change in apoptosis combined with chemotherapy was observed. (A) Protein expression of drug resistance genes (LRP, MRP and MDR1). (B) mRNA expression of drug resistance genes (LRP, MRP and MDR1). (C) Anti-oncogene expression including DAPK and IRF-1. (D) Cell cycle after culture with hBMSCs combined with Cy. (E) Cell apoptosis after culture with hBMSCs combined with cyclophosphamide. *P<0.05. LRP, low-density lipoprotein receptor; MDR1, multi-drug resistance gene; MRP, multidrug resistance-associated protein; DAPK, death-associated protein kinase; IRF-1, interferon regulatory factor-1; d, day; PI, propidium iodide; R+SUP-B15 cells, tyrosine kinase inhibitor-resistant SUP-B15 cells; hBMSCs, human bone marrow stromal cells; PI, propidium iodide.

profound effects on therapeutic efficacy (28-34). The present study showed that hBMSCs promoted the proliferation of TKI-resistant Ph^+ ALL by promoting cell cycle progression to the S+G2-M phase and increasing the expression of cyclins A, C, D1 and E, inhibited apoptosis by upregulating anti-apoptotic genes (BCL-2 and BCL-XL) and downregulating apoptotic genes (BCL-XS, Bax and caspases 3, 7 and 9), increased expression of the BCR-ABL gene and the Wnt signaling pathway-associated genes (Wnt, GSK-3β, β-catenin, E-cadherin and PI3K) and transcription factors (c-myc, EphB2, FGF20 and MMP7), increased expression of drug resistance genes (LRP, MRP and MDR1) and decreased the expression of anti-oncogenes (DAPK and IRF-1). In addition, no change in apoptosis was observed subsequent to combination treatment with Cy.

BMSCs provide the 'soil' for hematopoietic stem-progenitor cell homing, proliferation and differentiation. Stromal cells may play an important role in the mechanisms underlying drug resistance and apoptosis in hematological malignancies and solid tumors, depending on the tumor model (35-39). Resistance to TKI is a vital aspect in Ph^+ ALL due to BCR-ABL mutations (2-5). Previous studies have suggested that stromal cells may contribute to TKI-resistance in CML (21-25). The present findings showed that hBMSCs promoted the proliferation of TKI-resistant SUP-B15 cells and promoted cell cycle progression to the S+G2-M phase. Cyclins are markers of tumor proliferation (40). The present study found that hBMSCs increased the expression of cyclins A, B, D1 and E and gradually increased with increasing culture time. Apoptosis was also observed using Hoechst 33342-PI staining and anti-apoptotic and apoptosis-associated genes were detected. The fluorescence staining with Hoechst 33342-PI was found to gradually decrease in R+SUP-B15 cells cultured with hBMSCs. hBMSCs increased the expression of BCL-2 and BCL-XL, which are proteins associated with cell proliferation. hBMSCs also increased the expression of BCL-XS, Bax, and caspases 3, 7 and 9, which are associated with cell apoptosis. R+SUP-B15 cells cultured with hBMSCs gradually grew faster, as observed by scanning electron microscopy.

Wnt signaling can regulate the BCR-ABL gene and promote the survival and proliferation of CML stem-progenitor cells (25,41). The present findings demonstrated that hBMSCs promoted the expression of the Wnt and BCR-ABL genes. Additional investigation showed that hBMSCs promoted the expression of E-cadherin, GSK-3β, PI3K and β-catenin, which are associated with the Wnt signaling pathway. hBMSCs also promoted the expression of c-myc, EphB2, MMP7 and FGF20, which are transcription factors of the Wnt signaling pathways.

Drug resistance is associated with rapid relapse and mortality (42). The present study found that hBMSCs increased the expression of LRP, MRP and MDR1, which are associated with drug resistance. The cells also decreased the expression of anti-oncogenes DAPK and IRF-1. BMSCs from leukemia patients protect the leukemia cells from spontaneous and drug-induced apoptosis (43,44). The present study showed that hBMSCs increased the proportion of R+SUP-B15 cells in the S+G2-M phase of the cell cycle, and no change in apoptosis was observed when the chemotherapy drug was added to the medium.

Overall, the present study showed that hBMSCs can promote the growth of TKI-resistant Ph^+ ALL through promotion of the cell cycle, and the upregulation of cyclins, anti-apoptotic genes, BCR-ABL gene, Wnt signaling pathway-associated genes and transcription factors and drug resistance genes. Downregulation of apoptotic genes and anti-oncogenes also contributes to this. Therefore, targeting hBMSCs may be a promising approach to inhibit the growth of TKI-resistant Ph^+ ALL.

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