Expression and role of fibroblast activation protein α in acute myeloid leukemia

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Abstract. Currently, the prognosis of acute myeloid leukemia (AML) is poor. In the AML microenvironment, bone marrow (BM) mesenchymal stem cells (BMMSCs) serve an important role in protecting AML cells from chemotherapy-induced apoptosis. The present study aimed to evaluate the expression of fibroblast activation protein α (FAPα) in BMMSCs and BM biopsy samples via flow cytometry, reverse transcription-quantitative PCR and immunohistochemistry, as well as to identify the correlation between the expression of FAPα in BM with clinical parameters and survival of newly diagnosed patients with AML. Subsequently, the protective effect of FAPα on Cytosine arabinoside (Ara-C)-induced apoptosis in Kasumi-1 cells was investigated via small interfering (si)RNA, and its underlying mechanism was examined by western blotting. The results demonstrated significant differences in FAPα expression in BMMSCs and BM biopsy samples between patients with AML and healthy donors. Furthermore, BMMSCs protected Ara-C-induced Kasumi-1 cells from apoptosis, and knockdown of FAPα using siRNA decreased this protection. It was found that Kasumi-1 cells expressed β-catenin, which could be inhibited by Ara-C, and β-catenin expression was significantly activated when co-cultured with BMMSCs, even in the presence of Ara-C. Knockdown of FAPα with siRNA significantly suppressed the expression of β-catenin. The present results indicated that FAPα serves an important role in the AML BM microenvironment, and that increased expression of FAPα in BM may be a poor prognostic factor in patients with AML. Moreover, the current findings demonstrated that BMMSCs protected AML cells from apoptosis, which was in part contributed by FAPα, and may occur via the β-catenin signaling pathway.

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Introduction

Acute myeloid leukemia (AML) is a common hematologic malignancy with a statistical incidence rate of 38 case per million individuals in the United States between 2001 and 2007 (1). In the last 20 years, no significant improvement has been achieved in the prognosis or long-term outcomes of AML, and a considerable number of patients still experience induction failure or relapse after complete remission (CR) (2). The interaction between tumor cells and the tumor microenvironment is one of the key mechanisms of chemoresistance in tumor cells, including AML (3,4). Bone marrow (BM) mesenchymal stem cells (BMMSCs) serve an important role in protecting AML cells from chemotherapy-induced apoptosis (5,6). Previous studies have reported that tumor cells recruit BMMSCs by secreting cytokines or chemokines, and culturing BMMSCs to become cancer-related fibroblasts (CAFs) supports the progression of malignant cells (7,8). CAFs have been considered as central components in the tumor microenvironment and serve vital roles in tumor features, such as proliferation, angiogenesis, invasion and metastasis (9-11). However, the exact origin of CAFs remains unknown (12).

BMMSCs have recently been recognized as one of the origins of CAFs (13,14). However, a precise molecular definition of CAFs has not yet been elucidated. Previous studies have revealed that CAFs express cell surface markers, including fibroblast activation protein (FAPα) (15). Across a wide range of human cancer types, such as gastric carcinoma, breast cancer and colon cancer, the expression of FAPα has been reported to correlate with a higher tumor grade and worse overall survival in solid tumors (16-20). Considering the important role of FAPα in tumor progression and its rare expression in healthy tissues, FAPα has become a key target in tumor therapy (21,22). Previously, it was observed that tumor cells can reprogram BMMSCs to evolve into CAFs, thereby further promoting the progression of hematological malignancies (23). The expression of FAPα in BMMSCs moderately increases the number of tumor cells in conditioned medium from myeloma cells and those co-cultured with multiple

myeloma (MM) cells (24). Furthermore, knockdown of FAPα with small interfering (si)RNA decreases the protection of bortezomib-induced apoptosis in MM cells (24). However, it remains unknown whether the expression of FAPα in BMMSCs is different between patients with AML and healthy donors, nor has it been elucidated whether FAPα serves an important role in mediating AML cell features. Therefore, the aim of the present study was to investigate the expression of FAPα in BMMSCs and BM, as well as to identify the role of FAPα in BMMSCs in protecting AML cells from apoptosis.

Materials and methods

Cell culture. The human AML cell line, Kasumi-1, was obtained from the Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic. Cells were cultured in RPMI-1640 (Thermo Fisher Scientific, Inc.) medium containing 10% heat-inactivated FBS (Thermo Fisher Scientific, Inc.), 1% l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C and 5% CO_2.

BMMSCs were cultured according to a previously published paper (25). In total, 15 newly diagnosed patients with AML were selected, including six males and nine females (median age 56 years; age range 26-78 years). The inclusion criteria for patients with AML were as follows: i) Age ≥18 years old; ii) non-acute promyelocytic leukemia; ii) the diagnostic criteria were based on the 2016 World Health Organization diagnostic criteria for AML (26); and iv) no other malignant tumors-present, except AML. The control group consisted of healthy adults who were matched for age and sex with patients with AML. After informed consent was obtained, in keeping with ethical guidelines of The Second Affiliated Hospital of Nanchang University and the Declaration of Helsinki, BMMNCs were obtained from posterior superior iliac crest bone marrow of 15 healthy donors and 15 newly diagnosed patients with AML from The Second Affiliated Hospital of Nanchang University between January 2018 and January 2020 via lymphocyte separation medium (Tianjin Hao Yang Biological Manufacture Co., Ltd.), and were cultured. Half of the medium was replaced every 3-7 days. After culture for 2-4 weeks, cells became adherent and fibroblast-like, reaching >90% confluency. Then, cells were digested with 0.25% trypsin-EDTA and subcultured in RPMI-1640 (Thermo Fisher Scientific, Inc.) medium containing 10% heat-inactivated FBS (Thermo Fisher Scientific, Inc), 1% l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C and 5% CO_2. MSCs of passage 2-6 were used in experiments. Clinical and demographic parameters of 15 newly diagnosed patients with AML and healthy donors are presented in Table I.

Identification of BMMSCs. BMMSCs were detached from the culture flasks using Accutase™ solution (EMD Millipore). Cells were washed twice with PBS, non-specific antigens were blocked with 5% goat serum (Beijing Biosynthesis Biotechnology Co., Ltd.) for 1 h at room temperature, and then incubated with anti-CD14-FITC (cat. no. 561712; BD Pharmingen; BD Biosciences), anti-CD34-FITC (cat. no. 560942; BD Pharmingen; BD Biosciences), anti-CD90-APC (cat. no. 561971; BD Pharmingen; BD Biosciences), anti-CD105-PE (cat. no. 560839; BD Pharmingen; BD Biosciences) and anti-CD45-PE-Cy7 (cat. no. 560915; BD Pharmingen; BD Biosciences) antibodies for 15 min at 4˚C according to the manufacturer's instructions. Mouse IgG1K (cat. no. 562438; BD Pharmingen; BD Biosciences; 1:500) was incubated with cells at 4˚C as an isotype control. Cells were analyzed via flow cytometry (Gallios; Beckman Coulter, Inc.) and the FlowJo software program (FlowJo7.6; FlowJo LLC).

Detection of FAPα expression and myeloblasts in marrow via flow cytometry. BMMSCs were detached from culture flasks using Accutase™ solution, washed twice with PBS and non-specific antigens were blocked with 5% goat serum (Beijing Biosynthesis Biotechnology Co., Ltd.) for 1 h at room temperature. Then, cells were incubated with mouse anti-FAPα (cat. no. sc-100528; Santa Cruz Biotechnology, Inc.; 1:500) for 2 h at 4˚C. Subsequently, cells were washed twice with PBS and incubated with secondary antibody Alexa Fluor 488-labeled goat anti-mouse IgG (cat. no. A11001; Invitrogen; Thermo Fisher Scientific, Inc.; 1:500) for 30 min at 4˚C. Cells were analyzed via flow cytometry (Gallios; Beckman Coulter, Inc.) and the FlowJo7.6 software program (FlowJo LLC). The steps to detect the myeloblasts in marrow were as follows: Leukemia-related phenotype was assess using antibodies (CD7-FITC, cat. no. 561933; CD117-PE, cat. no. 562407; CD19-APC, cat. no. 560727; HLA-DR-PE, cat. no. 560651; CD15-FITC, cat. no. 560997; CD34-APC,cat. no. 560940; CD56-FITC, cat. no. 562794; CD13-PE, cat. no. 560998; CD11b-APC,cat. no. 561690; CD64-PE, cat. no. 561926; CD14-FITC, cat. no. 561712; CD33-APC, cat. no. 561817; cCD22-PE, cat. no. 563941; cCD3-APC, cat. no. 561800; BD Pharmingen; BD Biosciences; 1:500. MPO-FITC, cat. no. 130-107-177; Miltenyi Biotec; 1:500.), followed by the addition of 50-100 μl bone marrow. This was mixed well and incubated in dark at room temperature for 15-20 min. Then, 1 ml hemolysin was added and incubated in dark for 12 min. Centrifugation with 200 x g at room temperature for 5 min was conducted, the supernatant was removed and 300 μl PBS was added for suspension. Finally, flow cytometry (Gallios; Beckman Coulter, Inc.) was used for detection.

Immunofluorescence. Before use in experiments, BMMSCs were seeded (60%) in 6-well plates and cultured for 24 h. Then, cells were washed three times with PBS for 5 min each and fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were washed three times with PBS and permeabilized within 0.3% Triton X-100 for 10 min. After being washed three times with PBS, non-specific antigens were blocked with 5% goat serum (Beijing Biosynthesis Biotechnology Co., Ltd.) for 1 h at room temperature. After being washed three times with PBS, cells were and incubated with anti-FAPα (cat. no. sc-100528; Santa Cruz Biotechnology, Inc.; 1:500) at 4˚C overnight. Then, cells were incubated in the dark with Alexa Fluor 488-labeled goat anti-mouse IgG (cat. no. A11001; Invitrogen; Thermo Fisher Scientific, Inc.; 1:500) at 4˚C for 30 min. After being washed three times with PBS, cells were incubated with DAPI (1:500) for 5 min at room temperature in the dark. Then, cells were visualized using a fluorescence microscope with x10 and x40 magnification and images were collected.

Table I. Clinical and demographic parameters of participants in the study.

Parameter	Patients with acute myeloid leukemia	Healthy donors	P-value
Number (untreated)	15 (8)	15 (15)	>0.05
Age, years	56 (26-78)	47 (25-65)	>0.05
Sex			>0.05
Male	6	7	
Female	9	8	
FAB system classification			
M2	9	0	
M4	2	0	
M5	3	0	
M6	1	0	
WBC, 10^9/l	21.28 (1.03-39.04)	5.67 (3.34-9.67)	<0.05
HGB, g/l	83 (36-150)	128 (115-158)	<0.05
PLT, 10^9/l	32 (5-124)	198 (119-279)	<0.05
Myeloblasts in marrow, %	50±22.55	-	-

Data are presented as the number, median and interquartile range or mean ± SD. FAB, French-American-British; WBC, white blood cell; HGB, hemoglobin; PLT, platelet.

Immunohistochemistry. A small piece of cylindrical bone marrow tissue, 0.5-1 cm long, was collected from the puncture needle. After sampling, tissues were immediately placed into 10% neutral buffered formalin solution and fixed at room temperature for 6-24 h. Then, 4% bone marrow decalcification solution (Vignes; http://www.wexisgp.com/) was used for 2 h at 37˚C. The slices were prepared with a thickness of 3 μm and baked at 68˚C for 1 h. The antigen was dewaxed with xylene and then repaired with citric acid antigen repair solution (cat. no. G1202; Wuhan Servicebio Technology Co., Ltd.). The slices were placed into 3% hydrogen peroxide solution (cat. no. G0115; Wuhan Servicebio Technology Co., Ltd.), incubated at room temperature and protected from light for 25 min to block endogenous peroxidase. Then, 3% BSA (cat. no. G5001, Wuhan Servicebio Technology Co., Ltd.) was added to evenly cover the tissue and slices were sealed at room temperature for 30 min. Slices were incubated overnight with anti-FAPα antibody (cat. no. ab53066; Abcam; 1:100) at 4˚C. Then, slices were incubated with a secondary goat anti-rabbit antibody (cat. no. GB23303; Wuhan Servicebio Technology Co., Ltd.; 1:200) at room temperature for 50 min. After DAB (cat. no. G1211; Wuhan Servicebio Technology Co., Ltd.) color development, re-dyeing nucleus with hematoxylin for 3 min at room temperature, dehydration and sealing with neutral gum, images were captured using Nikon E100 light microscope with x200 and x400 magnification and analyzed using Image-pro plus 6.0 (Media Cybernetios, Inc; Version 6.0.0.260 for windows 2000/XP professional; serial no. 41M60032-00032) [optical density (OD)/area].

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from BMMSCs using RNAiso™ Plus (Takara Bio, Inc.) according to the manufacturer's instructions. cDNA was generated from 1 μg total RNA using Prime Script™ RT reagent kit (Perfect Real Time; Takara Bio, Inc.) with DNA Eraser according to the manufacturer's instructions. Duration of RT was 15 min at 37˚C. RT-qPCR was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio, Inc.) following the manufacturers' instructions on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). GAPDH served as an endogenous control. The primer sequences for FAPα and GAPDH amplification were as follows: FAPα forward, 5'-GTATTTGGAGTTGCCACCTCTG-3' and reverse, 5'-GAAGGGCGTAAGACAATGCAC-3'; and GAPDH forward, 5'-AAGGTGAAGGTCGGAGTCAAC-3' and reverse, 5'-GGGGTCATTGATGGCAACAATA-3'. The thermocycling conditions for FAPα and GAPDH amplification were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The primer specificity was verified using the melt curve. The relative quantification of FAPα in BMMSCs was calculated using the $2^{-\Delta\Delta Cq}$ method (27).

Transfection and cell apoptosis analysis via flow cytometry. The day before transfection, BMMSCs were seeded (60%) in 12-orifice plates. When the cells reached ~90% confluency, they were transfected with FAPα small interfering (si) RNA (forward, 5'-CGCCCUUCAAGAGUUCAUATT-3' and reverse, 5'-UAUGAACUCUUGAAGGGCGTT-3'), FAPα-Mock (only lipo2000 and no siRNA sequence) and NC siRNA (forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3') at room temperature using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The mass and concentration of transfected siRNA was 5 μl and 100 pmol, respectively. After 6 h, the medium was removed, and Kasumi-1 cells were added and co-cultured with transfected BMMSCs. The co-culture ratio of AML cells and BMMSCs was 1-2:1.

A total of 100 μM Cytosine arabinoside (Ara-C; Pfizer, Inc.) was added to the well to induce apoptosis in Kasumi-1 cells (28) for 24 h at room temperature. After 24 h, co-cultured Kasumi-1 cells were isolated from the monolayer of BMMSCs, and gently transferred into the monolayer with medium. Next, cells were stained with FITC-conjugated Annexin V and PE-conjugated PI at 4˚C for 30 min following the Annexin V FITC Apop Dtec Kit I (BD Biosciences) manufacturer's instructions. Annexin V-FITC and PI-PE positive cells were considered apoptotic cells. The interfering effect was determined via RT-qPCR and flow cytometry (Gallios; Beckman Coulter, Inc.) and the FlowJo7.6 software program (FlowJoLLC).

Western blot analysis. After transfection of BMMSCs with FAPα-siRNA, BMMSCs ($2x10^5$) and Kasumi-1 cells ($5x10^5$) were cocultured in 6-well plates. After 48 h, Kasumi-1 cells were collected and washed twice with PBS. Total cell lysates were obtained using lysis buffer (Beyotime Institute of Biotechnology) supplemented with 1 mM PMSF. The protein concentration was determined using the BCA (Sangon Biotech Co., Ltd.) method according to the manufacturer's

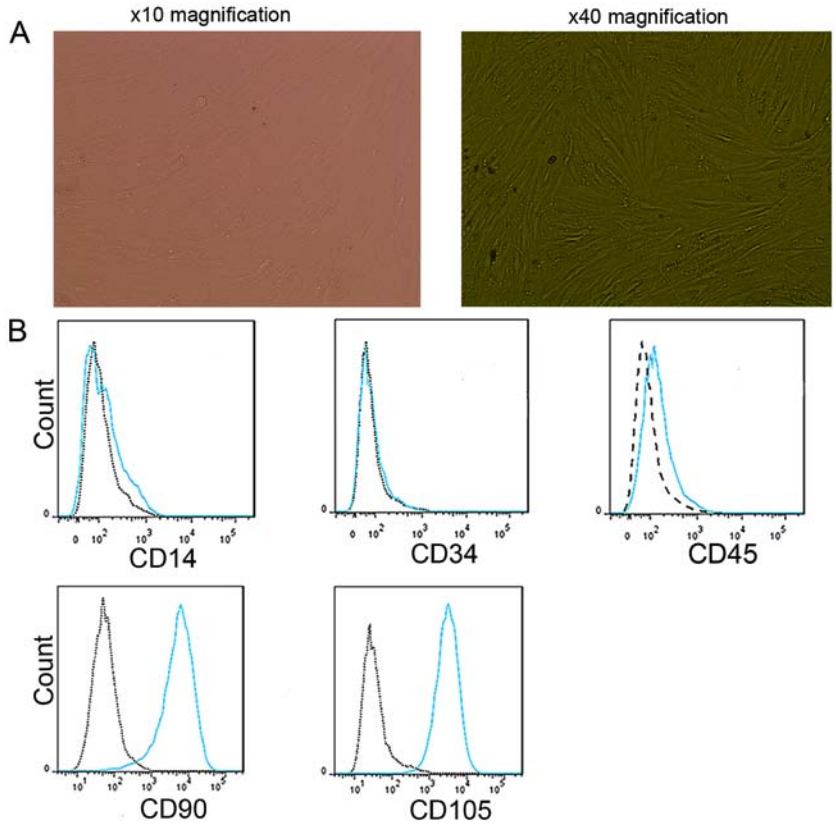

Figure 1. Phenotype of BMMSCs. (A) BMMSCs had a fibroblast-like morphology and adhered to the flask. Magnification, x10 and x40. (B) BMMSCs had a high expression of CD90 and CD105, and a low expression of CD14, CD34 and CD45. BMMSCs, bone marrow mesenchymal stem cells.

guidelines. An equal amount of protein (20-40 g) was separated via 10-12% SDS-PAGE and transferred to PVDF membranes. Next, membranes were blocked with 5% non-fat milk for 2 h at room temperature. PVDF membranes were incubated with anti-actin and anti-β-catenin antibodies (cat. no. 2698S; Cell Signaling Technology, Inc.; 1:500) overnight at 4˚C. Subsequently, membranes were washed and incubated with a horseradish peroxidase-conjugated antibody (cat. no. 7074S; Cell Signaling Technology, Inc.; 1:5,000) in 0.2% TBS-Tween at room temperature for 2 h. After being washed, protein bands were visualized using an ECL detection kit (Biological Industries). Semi-quantification of the western blotting was performed using the Bio-Rad imaging system and ImageJ software (29) (Life-Line Fiji versions java8; https://imagej.net/Fiji/Downloads) to detect protein expression.

Statistical analysis. All the experiments were repeated three times. All analyses were performed using SPSS 22.0 software (IBM Corp.). The χ^2 test was used for categorical data. Measurement data were presented as mean ± SD. The unpaired Student's t-test was used for two independent groups. Multiple comparisons were performed using one-way ANOVA (Bonferroni) statistical analysis. Pearson correlation test was used for correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of BMMSCs. The experimental results demonstrated that cultured BMMSCs adhered to the flasks and had a fibroblast-like morphology (Fig. 1A). BMMSCs of passage 2 had a low expression of CD14, CD34 and CD45, and a high expression of CD90 and CD105 (n=3). Analysis of these CD molecular markers on BMMSCs via flow cytometry is presented in Fig. 1B.

Expression of FAPα in BMMSCs of newly diagnosed patients with AML and healthy donors. Significant differences in the expression of FAPα between newly diagnosed patients with AML and healthy donors were identified via flow cytometry (70.92±4.38 vs. 36.74±10.37%, respectively; P=0.0072; Fig. 2B and C), RT-qPCR (24.75±2.75 vs. 9.77±1.94, respectively; P=0.0001; Fig. 2D) and immunofluorescence (Fig. 2A).

Expression of FAPα in BM biopsy samples of newly diagnosed patients with AML and healthy donors. The expression of FAPα in BM biopsy samples between newly diagnosed patients with AML and healthy donors was examined via immunohistochemistry. FAPα expression was observed in the BM mesenchymal matrix as indicated by the positively stained yellow area (Fig. 3A and B). Image-pro plus 6.0 software was used to estimate the percentage of the stained area, represented as OD/area. The OD/area value was higher in AML BM compared with BM from healthy donors (OD/area, 11.88±4.55 vs. 5.16±3.67; P<0.0001; n=15; Fig. 3C).

Correlation of FAPα expression in BM with clinical parameters and survival time of newly diagnosed patients with AML. The correlation between FAPα expression in BM and

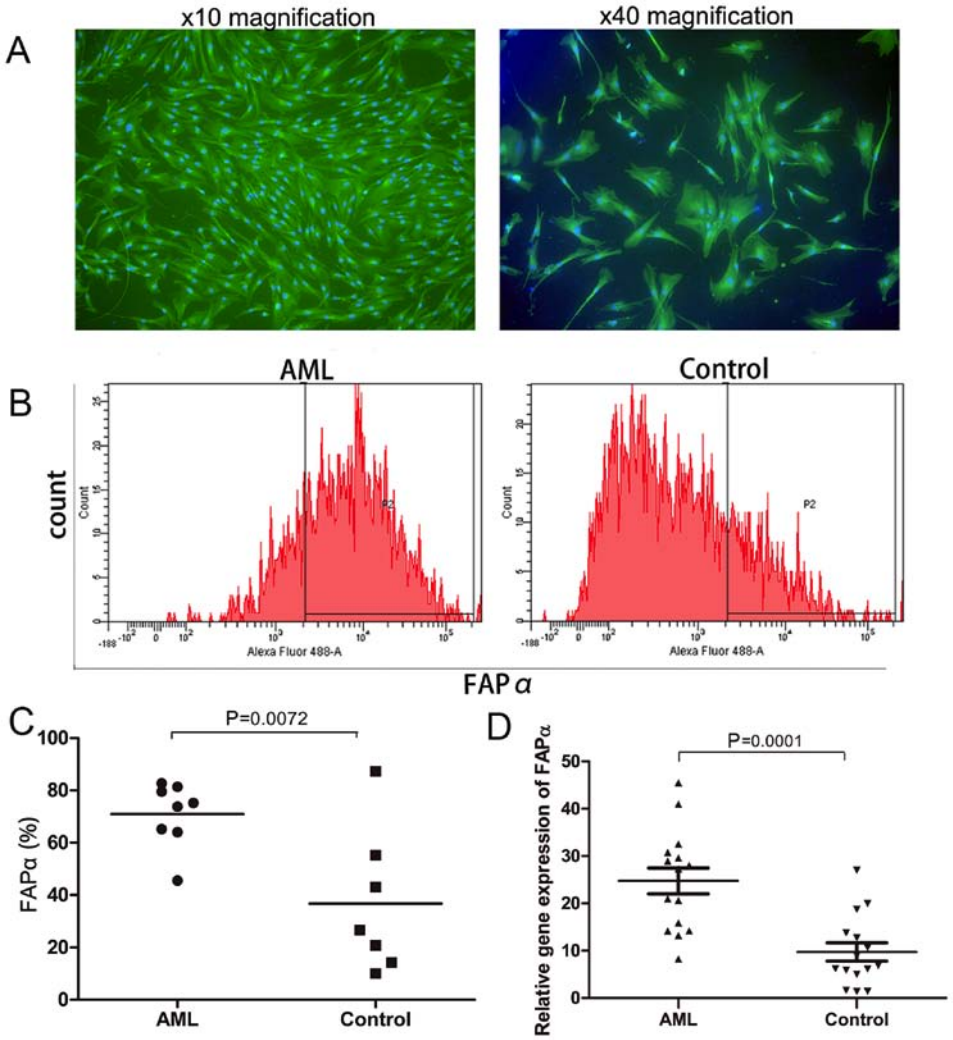

Figure 2. Expression of FAPα on BMMSCs. (A) FAPα expression in BMMSCs detected via immunofluorescence. Magnification, x10 and x40. (B) A representative analysis of FAPα expression in BMMSCs detected via FCM. (C) Expression of FAPα in BMMSCs detected via FCM and (D) reverse transcription-quantitative PCR. FCM, flow cytometry; BMMSCs, bone marrow mesenchymal stem cells; FAPα, fibroblast activation protein α; AML, acute myeloid leukemia.

clinically parameters of newly diagnosed AML patients was analyzed, and the results demonstrated that the OD/area value of FAPα was positively correlated with the proportion of myeloblasts in marrow detected via flow cytometry (r=0.878; P<0.0001; Fig. 4A). No significant correlation was observed between the OD/area value of FAPα and leukocytes (r=0.444; P=0.0976; Fig. 4B), hemoglobin (r=0.331; P=0.2287; Fig. 4C) or platelets (r=0.278; P=0.3153; Fig. 4D). Representative flow scatter plots of myeloblasts in marrow is presented in Fig. 4E. The OD/area value of FAPα was significantly and negatively correlated with survival in eight patients who did not undergo chemotherapy (r=0.815; P=0.0137; Fig. 4F).

BMMSCs protects AML cells from Ara-C-induced apoptosis, which in part is contributed by FAPα. RT-qPCR was used to verify successful transfection prior to detecting apoptosis. The relative expression of FAPα mRNA in the FAPα-siRNA group was significantly lower compared with that in negative control (NC)-siRNA group (0.11±0.01 vs. 0.84±0.08; P=0.0001; Fig. 5A). Then, flow cytometry results demonstrated that BMMSCs protected Kasumi-1 cells from apoptosis, even in the presence of Ara-C (100 μM), and knockdown of FAPα with siRNA decreased the protective effect of BMMSCs (Fig. 5C). The proportion of apoptotic cells in the MSC-Mock group was significantly lower compared with that in the Ara-C group (12.96±0.95 vs. 25.66±1.54%; P<0.001; n=9; Fig. 5B). Knockdown of FAPα using siRNA decreased the protective effect. The proportion of apoptotic cells in the FAPα-siRNA group was significantly increased compared with the proportion in the NC-siRNA group (22.69±1.99 vs. 13.29±1.10%; P<0.001; n=9; Fig. 5B). The proportion of apoptotic cells in FAPα-siRNA group was also lower compared with that in the Ara-C group (22.69±1.99 vs. 25.66±1.54%; P=0.001; Fig. 5B).

Possible mechanism of FAPα protecting AML cells from apoptosis. The results demonstrated that Kasumi-1 cells expressed β-catenin, which could be inhibited by Ara-C. The expression of β-catenin in Kasumi-1 cells was significantly increased when Kasumi-1 cells were cocultured with BMMSCs and NC-siRNA for 48 h, and even in the presence of Ara-C the expression of β-catenin is activated. Compared with NC-siRNA, knockdown of FAPα using siRNA significantly suppressed the expression of β-catenin (Fig. 6A). The experiment was performed in triplicate, the relative expression of β-catenin

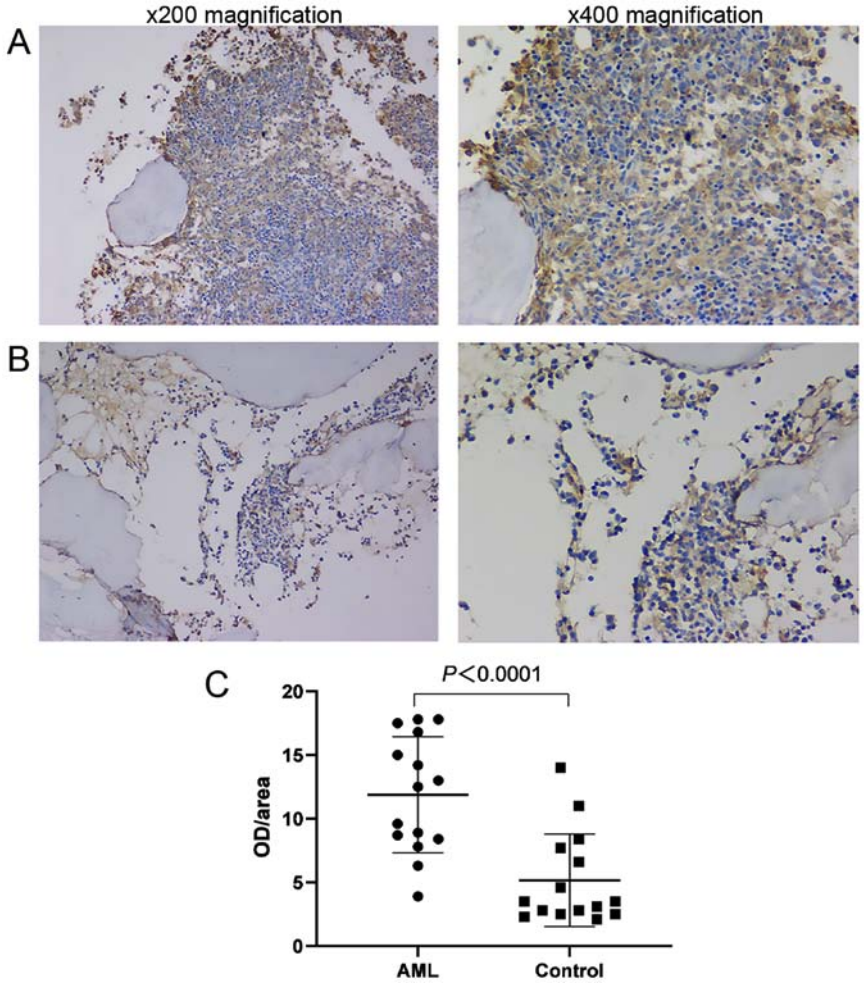

Figure 3. Expression of FAPα in BM biopsy samples was detected via immunohistochemical staining. FAPα expression was observed in the BM mesenchymal matrix by the positively stained yellow area in a (A) patient with AML and a (B) healthy donor. Magnification, x200 and x400. (C) Image-pro plus 6.0 software was used to estimate the percentage of the stained area, represented as OD/area (P<0.0001; n=15). OD, optical density; FAPα, fibroblast activation protein α; AML, acute myeloid leukemia; BM, bone marrow.

was calculated and statistical analyses were performed. In the absence of BMMSCs, the expression of β-catenin was decreased from 113.20±2.77 to 44.02±0.06 in groups with and without Ara-C, respectively (P<0.0001; Fig. 6B). In addition, in the presence of Ara-C and BMMSCs, the expression of β-catenin in the NC-siRNA group and FAPα-siRNA group was 116.84±0.40 and 82.11±1.26, respectively (P<0.0001; Fig. 6B).

Discussion

In 1991, Caplan's pioneering work reported the existence of BMMSCs (30). Typically, MSCs are isolated by their ability to adhere to the plastic surface of a culture dish, which demonstrates the ability of the cells to expand in culture plates, and MSCs have specific markers of immunological characteristics (31). The International Society for Cellular Therapy suggests three minimal criteria for the characterization of MSCs: i) Adherence to plastic; ii) expression of markers associated with MCS (such as CD73, CD90 and CD105), and the lack of hematopoietic-related cell expression (including CD34, CD45, CD11b or CD14, CD19 or CD79 and human leukocyte antigen DR); and iii) potential tri-lineage differentiation into adipocytes, osteoblasts and chondrocytes (30). In the present study, cultured BMMSCs adhered to culture flasks and were fibroblast-like in morphology. In addition, BMMSCs of passage 2 had a low expression of CD14, CD34 and CD45, and high expression of CD90 and CD105. Collectively, the current findings met the minimal criteria for defining MSCs.

FAPα (also known as seprase) is a serine oligopeptidase that was originally identified in 1986 as an inducible cell surface glycoprotein F19 (32), and was renamed FAP in 1994 based upon its abundance in activated fibroblasts (33). Human FAP is comprised of M(r) 95,000 (p95, FAPα) and M(r) 105,000 (p105, FAPβ) subunits, which are conjugated by non-covalent, non-disulfide bonds (34). Immunoblot experiments have shown that FAPα, but not FAPβ, carries an epitope that is bound by monoclonal antibody F19 (35), and so the F19 surface antigen was renamed to FAPα.

Most healthy adult tissues have little or no detectable FAPα expression (36). However, FAPα expression is highly upregulated during active tissue remodeling of fibroblasts, including wound healing, fibrosis and at cancer sites (37). FAPα is also expressed in BMMSCs, and promotes the motility of human BMMSCs (38). In the context of cancer, FAPα has been widely considered as a marker of CAFs, which have multiple pro-tumorigenic functions (38). Moreover, FAPα itself has

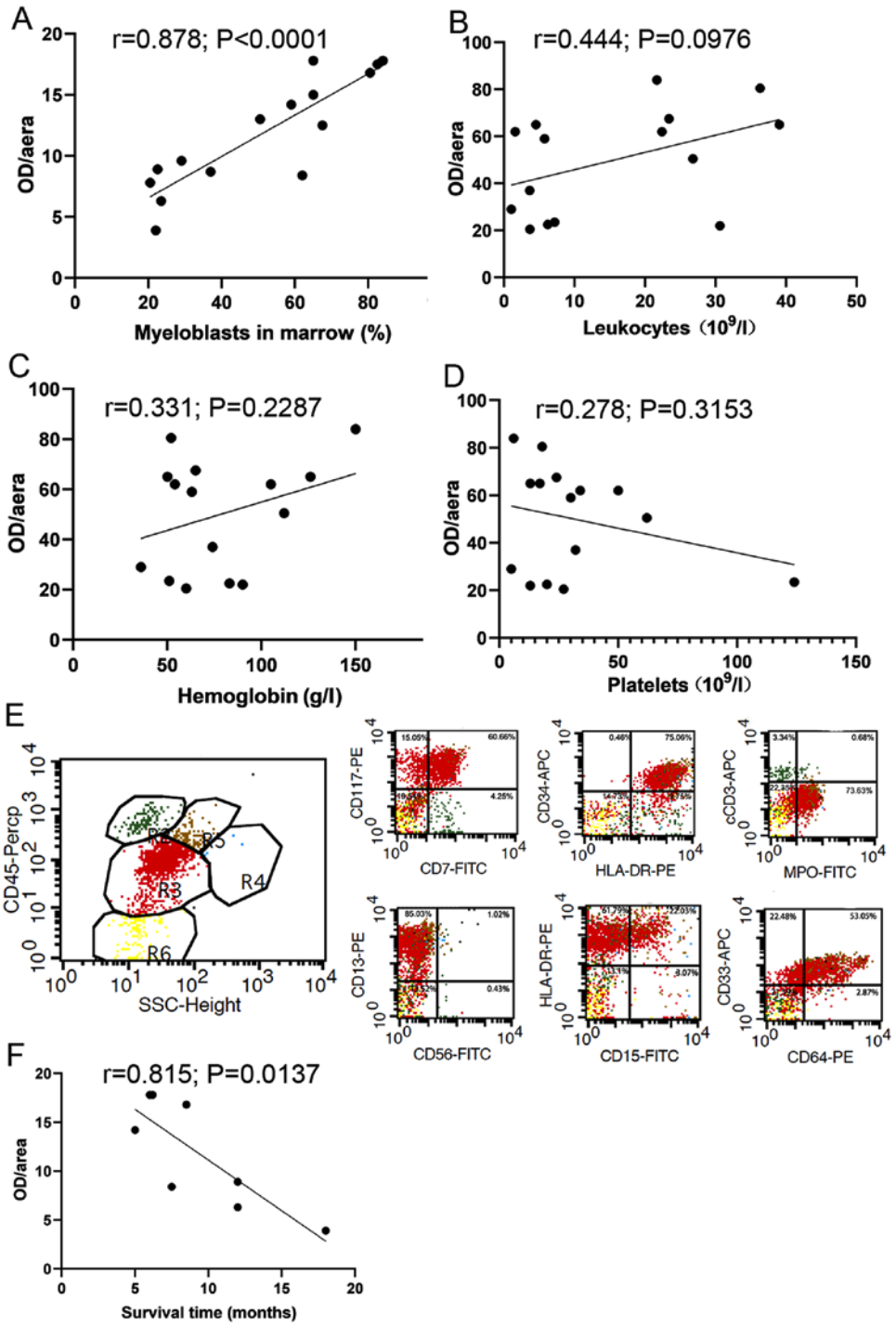

Figure 4. Correlation between the expression of FAPα in BM with clinical parameters and survival of newly diagnosed patients with AML. (A) OD/area value of FAPα positively correlated with the proportion of myeloblasts in marrow detected via flow cytometry (r=0.878, P<0.0001). No significant correlation was observed between the OD/area value of FAPα and (B) leukocytes (r=0.444; P=0.0976), (C) hemoglobin (r=0.331; P=0.2287) or (D) platelets (r=0.278; P=0.3153). (E) Representative flow scatter plots of myeloblasts in marrow. (F) OD/area value of FAPα was significantly and negatively associated with survival in eight patients who did not undergo chemotherapy (r=0.815, P=0.0137). OD, optical density; FAPα, fibroblast activation protein α; AML, acute myeloid leukemia; BM, bone marrow.

been reported to exert pro-tumorigenic activity, both via enzymatic and non-enzymatic mechanisms (36,39). Previous studies have revealed that tumor cell conditioned medium can transform BMMSCs into CAFs, thereby promoting tumor progression (40). Moreover, tumor cells can reprogram BMMSCs and promote the evolution of BMMSCs into CAFs, further facilitating the development of hematological malignancies (41). Therefore, it was hypothesized that the AML microenvironment may instruct BMMSCs to become CAFs, to further increase the expression of FAPα in BMMSCs compared with BMMSCs in a healthy environment.

The present study first evaluated the expression of FAPα on BMMSCs between newly diagnosed patients with AML and healthy donors using RT-qPCR and flow cytometry. The experimental results demonstrated that the expression of FAPα in newly diagnosed patients with AML was significantly

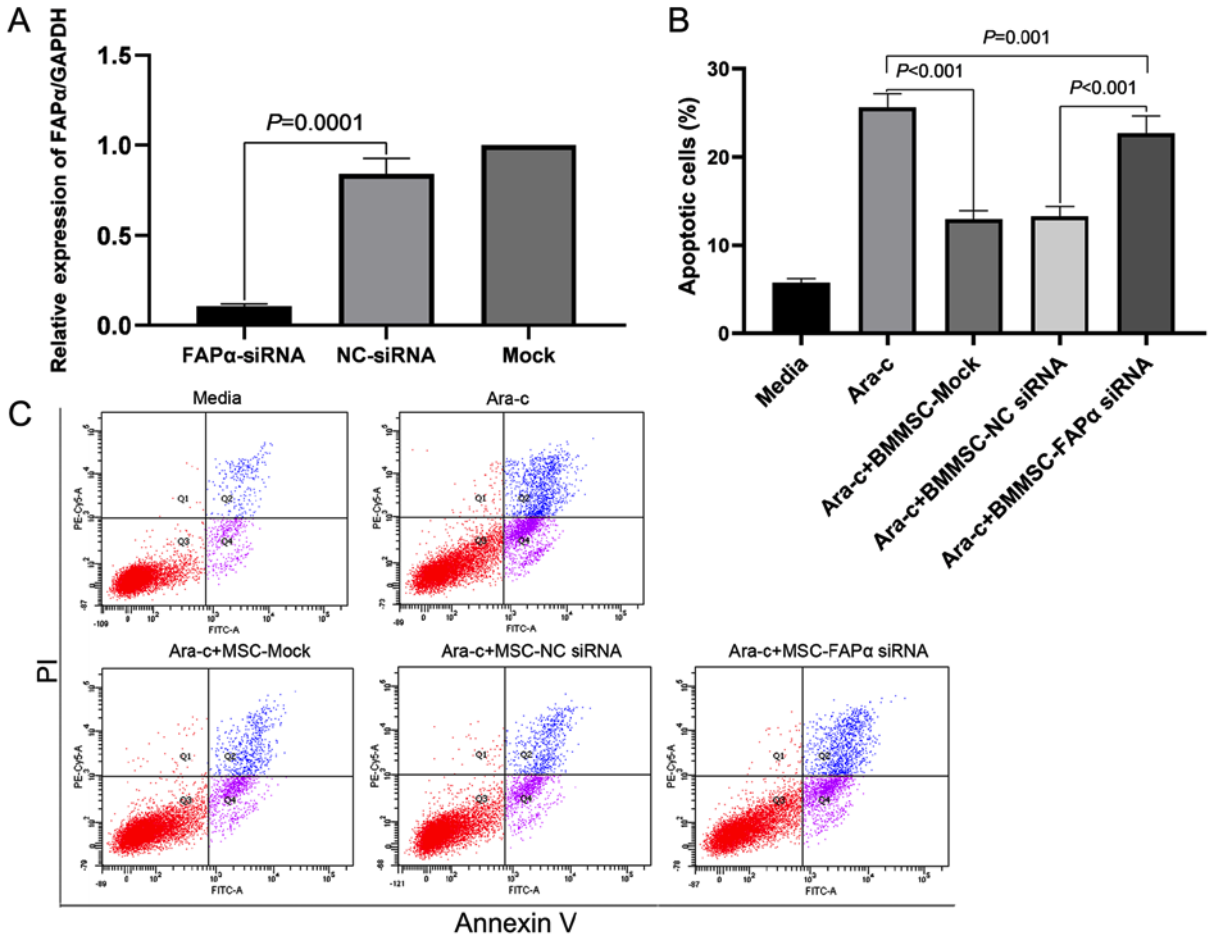

Figure 5. BMMSCs protect AML cells from Ara-C-induced apoptosis, which is partly contributed by FAPα. (A) Verification of successful transfection using reverse transcription-quantitative PCR. The relative expression of FAPα mRNA in FAPα-siRNA group was significantly lower compared with that in NC-siRNA group (0.11±0.01 vs. 0.84±0.08; P=0.0001). (B) Proportion of apoptotic cells in the BMMSC-Mock group was significantly lower compared with that of the Ara-C group (12.96±0.95 vs. 25.66±1.54%; P<0.001; n=9). Knockdown of FAPα using siRNA decreased the protective effect. The proportion of apoptotic cells in the FAPα-siRNA group was significantly increased compared with the proportion in the NC-siRNA group (22.69±1.99 vs. 13.29±1.10%; P<0.001; n=9). The proportion of apoptotic cells in FAPα-siRNA group was lower compared with that in the Ara-C group (P=0.001). (C) A representative apoptosis analysis of Kasumi-1 in coculture system with FAPα knockdown or not in the presence of 100 μM Ara-C detected via FCM. Annexin V-FITC and PI-PE positive cells were considered apoptotic cells. NC, negative control; siRNA, small interfering RNA; BMMSCs, bone marrow mesenchymal stem cells; FAPα, fibroblast activation protein α; Ara-C, Cytosine arabinoside.

higher compared with healthy donors. However, the specific origin of FAPα within the BM remains to be elucidated. To further confirm the expression of FAPα in BM, the expression of FAPα in the BM mesenchymal matrix was positively stained yellow. It was identified that the OD/area value of FAPα in newly diagnosed patients with AML was significantly higher compared with that of healthy donors. Previous studies have reported that FAPα expression is associated with worse prognosis in solid tumors (42). However, the relationship between FAPα expression and the prognosis in hematologic tumors remains unknown. The present study demonstrated that the OD/area value of FAPα was positively correlated with the proportion of myeloblasts in BM as detected via flow cytometry, and was significantly negatively associated with survival time in eight patients who did not undergo chemotherapy due to the costs and risks. Collectively, these results suggested that FAPα serves an important role in the AML BM microenvironment, and may be involved in the development of AML. Thus, increased expression of FAPα in BM may be a poor prognostic factor in AML.

The interaction between AML cells and BMMSCs, either via direct contact or secreted cytokines, can promote AML progression, survival, growth, chemotherapy resistance and angiogenesis (43-46). However, little is known regarding the function of FAPα expression in BMMSCs and how it affects the features of AML cells. In the current study, flow cytometry results demonstrated that BMMSCs protected Ara-C (100 μM)-induced Kasumi-1 cell apoptosis in a cocultured system. Furthermore, knockdown of FAPα with siRNA decreased this protective effect. However, the proportion of apoptotic cells in the FAPα-siRNA group was lower compared with that in Ara-Cgroup. Thus, these results suggested that BMMSCs in the BM microenvironment of patients with AML were instructed to become CAFs to protect AML cells from apoptosis, which, in part was contributed by FAPα. However, other factors may be involved in the protective effect of BMMSCs on leukemia cell apoptosis.

Previous studies have revealed that, via a variety of mechanisms, BMMSCs inhibit AML cell apoptosis and promote their survival, proliferation and chemotherapy

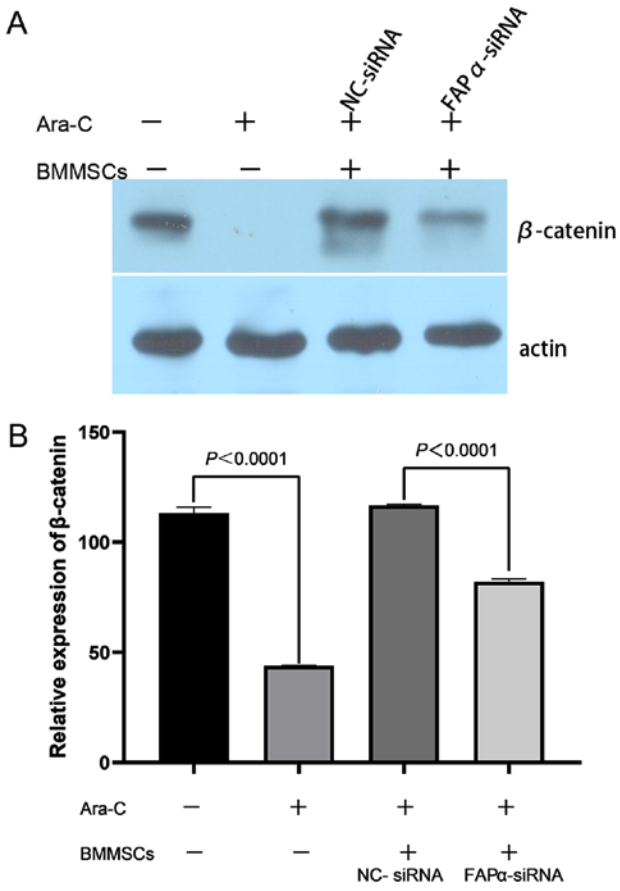

Figure 6. Possible role of FAPα in BMMSCs-mediated protection of AML cell line. (A) Kasumi-1 cells expressed β-catenin, which can be inhibited by Ara-C. The expression of β-catenin of Kasumi-1 cells was significantly activated when Kasumi-1 cells cocultured with BMMSCs. Knockdown of FAPα using siRNA significantly suppressed the expression of β-catenin. (B) In the absence of BMMSCs, the expression of β-catenin in groups with Ara-C was 113.20±2.77 and that without Ara-C was 44.02±0.06 (P<0.0001). In addition, in the presence of Ara-C and BMMSCs, the expression of β-catenin in the NC-siRNA group and FAPα-siRNA group was 116.84±0.40 and 82.11±1.26, respectively (P<0.0001). NC, negative control; siRNA, small interfering RNA; BMMSCs, bone marrow mesenchymal stem cells; FAPα, fibroblast activation protein α; Ara-C, Cytosine arabinoside.

resistance (46-51). In addition, it has been reported that Wnt ligands are the key drivers of most types of tissue stem cells in adult mammals, and mutated Wnt pathway components are causative in the progression of multiple growth-related pathologies and cancer (52). For instance, the Wnt/β-catenin pathway is required for the development of leukemia stem cells in AML (53). However, the current understanding of the activation of the BMMSC-mediated Wnt/β-catenin signaling pathway is limited. Furthermore, the relationship between FAPα and Wnt/β-catenin for AML cellular features remains unknown. The present study demonstrated that Kasumi-1 cells expressed β-catenin, which could be inhibited by Ara-C. However, the expression of β-catenin in Kasumi-1 cells was significantly increased when Kasumi-1 cells were cocultured with BMMSCs, even in the presence of Ara-C. The data also indicated that knockdown of FAPα using siRNA significantly suppressed the expression of β-catenin. Thus, it was suggested FAPα may be involved in BMMSC-mediated apoptosis of AML cells induced by Ara-C, which may be achieved via the β-catenin pathway.

In conclusion, the present results indicated that FAPα may serve an important role in the AML BM microenvironment and that it may be involved in the development of AML. Moreover, it was suggested that increased expression of FAPα in BM may be a poor prognostic factor in AML. Further studies demonstrated that BMMSCs could protect AML cells from apoptosis, which in part was contributed by FAPα, and likely occurs via the β-catenin signaling pathway. However, whether the activation of β-catenin was dependent on direct contact with BMMSCs or secretory of cytokines requires further research. In addition, the quantity of the samples should be expanded to clarify the relationship between FAPα expression and the prognosis of AML, and additional studies should be performed using other AML cell lines to further verify the anti-apoptotic role of FAPα in AML cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SM, FZ and GC designed the research and analyzed the data. SM, YZ and LY performed the cell function experiments. SM and YZ collected the BM liquid and biopsy samples, and reviewed the clinical features. SM prepared the images and drafted the original manuscript. FZ and GC reviewed and edited the manuscript. All authors read and approved the manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the Medical Research Ethics Committee of the Second Affiliated Hospital of Nanchang University. All patients and healthy donors provided written informed consent. The study in keeping with ethical guidelines of the Second Affiliated Hospital of Nanchang University and the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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