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The effects of legumain in THP1 leukemia cells

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Abstract: Legumain is a C13 family cysteine protease. It plays diverse roles under both physiological and pathological conditions. The high-level expression of legumain is detected in solid tumors. Legumain promotes the proliferation and migration of tumor cells. However, the effect of legumain in blood diseases has not been established. In this report, we studied the effect of legumain on leukemia cells by overexpressing it in THP1 cells. The results demonstrated that legumain promoted cell proliferation, whereas it had little effect on cell apoptosis. Furthermore, legumain promoted the migration of THP1 cells. It was worth noting that legumain decreased the stemness of THP1 cells. Further evidence showed that legumain decreased the expression of *Oct4*, *Sox2*, *Myc* in THP1 cells. Our study reveals the multifaced effects of legumain in leukemia cells and broadens the knowledge of legumain in malignancies.

Introduction

Legumain is a cysteine protease that belongs to the C13 family of cysteine proteases. It is also called asparagine endopeptidase (AEP) because of its specificity for asparagine bond (Chen *et al.*, 1997). Legumain is widely distributed among species, such as plants, invertebrate parasites as well as mammals. It is well conserved in human and murine (Barrett and Rawlings, 2001). In mammals, legumain has a broad range of tissue and cellular distribution. It is most abundant in kidney and testis (Chen *et al.*, 1997), whereas it can be detected in different cell types including bone marrow stromal cells, monocytes, adipocytes, and macrophages (Jafari *et al.*, 2017; Solberg *et al.*, 2015). Legumain also has a different subcellular distribution. It is mainly located in acid lysosomes, where the activation of the protease takes place, and it can also be detected in extracellular fluids such as serum (Lunde *et al.*, 2017; Smith *et al.*, 2012).

Legumain is involved in many key cellular pathways and biological processes under physiological conditions. Legumain plays an important role in the formation of MHC class II complex by removing the invariant chain chaperone of the MHC class II (Manoury *et al.*, 2003). It also influences the processing of antigens for MHC class II presentation in antigen-presenting cells (Dall and Brandstetter, 2016). It also supports human Th1 cell induction and activates the CTSL-C3-IFN-γ signal axis in human $CD4^+$T cells (Freeley *et al.*, 2018).

Legumain is involved in different pathological processes as well. Legumain plays a role in neuroinflammation in cognitive impairment. Knockout of legumain reduces the level of neuroinflammation, so as to improve cognitive impairment in stressed mice (Lian *et al.*, 2019). Legumain is involved in the induction of atherosclerotic vascular remodeling (Ozawa *et al.*, 2019). Previous studies showed that legumain was overexpressed in different human solid tumors, and overexpression of legumain promoted the proliferation, migration, and metastasis of tumor cells (Liu *et al.*, 2003). Furthermore, the high-level expression of legumain is related to poor prognosis and clinical stage in solid tumors (Zhen *et al.*, 2015).

Leukemia is a rapidly progressing hematopoietic malignancy (Thomas and Majeti, 2017). Both intrinsic and extrinsic factors play important roles in the transformation and progression of leukemia (Wang *et al.*, 2018a; Yang *et al.*, 2018b). However, the effect of legumain, either as an intrinsic factor or an extrinsic factor in blood diseases, especially leukemia, has not been documented. In this study, we explored the effects of overexpressed legumain in leukemia cells by constructing THP1 cells overexpressing legumain. Overexpression of legumain promoted the proliferation and migration of THP1 cells, whereas decreased the stemness of THP1 cells.

Materials and Methods

Cell lines and reagents

THP1 and HEK293T cells were purchased from American Type Culture Collection. Lentivirus vector pLV-EF1α-MCS-IRES-Bsd was obtained from Boisettia Inc (San Diego, CA). Fetal bovine serum (FBS), trypsin, penicillin/streptomycin,

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OPTI-MEM, RPMI 1640, DMEM, sodium pyruvate, MEM NEAA, and L-glutamine were purchased from Gibco (USA). SYBR Green PCR kit was purchased from Takara (Japan). *Xba* I and *Nhe* I restriction endonucleases were obtained from New England BioLabs (UK). H4434 was purchased from Stem Cell Technologies (Vancouver, BC, Canada). Annexin V/PI kit was purchased from BioLegend (San Diego, CA). Transwell chambers were purchased from Millipore (Bedford, MA).

Cell culture

All cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. Parental THP1 cells and infected THP1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin. HEK293T cells were cultured in DMEM medium supplemented with 10% FBS, sodium pyruvate, MEM NEAA, and L-glutamine. Logarithmic growth cells were used for subsequent experiments.

Construction of legumain expressing vector

PCR primers (Forward: 5'-GGCCTCTAGAGCCACCATGGT-TTGGAAAGTAGCTGTATTCCTCAGT-3', Reverse: 5'-GGC-CGCTAGCTCAGTAGTGACCAAGGCACACGTGGTCCAT-3') were designed according to the coding sequence (CDS) of human legumain (NCBI: NM_005606.7). The purified PCR product was digested by *Xba I* and *Nhe I* before inserted into the lentivirus vector pLV-EF1α-MCS-IRES-Bsd.

Construction of THP1 cells overexpressing legumain

The THP1 cell line overexpressing legumain was constructed using the lentivirus system following standard protocols. Briefly, HEK293T cells were transfected with packaging plasmids along with blank or recombinant plasmids using X-termeGENE HP DNA Transfection Reagent (Roche, USA). Lentivirus was collected 48 h later. THP1 cells were mixed with lentivirus and polybrene (8 μg/mL), followed by spinning at 1800 rpm for 90 min. Then cells were cultured in a lentivirus-free medium for 48 h. Finally, antibiotic blasticidin S (Bsd) was added at a concentration of 10 μg/mL to screen infected cells.

Flow cytometry analysis

BD CantoII flow cytometer (BD Biosciences) was used for FACS analysis. All experiments were conducted according to standard protocols. Data analysis was carried out using Diva (BD Biosciences, San Jose, CA) or FlowJo VX (Tree Star, San Carlos, CA).

Quantitative real time-PCR (qPCR)

Cells were collected, and total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. RNA concentration was determined, and cDNA was synthesized using M-MLV First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA) with oligo (dT) primer. qPCR was performed using SYBR Green Kit. The expression level of target genes was analyzed by the RQ value calculated through normalization to the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the ΔΔCt method. All primer sequences are listed in Tab. 1.

Cell growth assays

For the cell counting assay, 5×10^4 cells were seeded per well in a 24-well plate. The cell number each well was counted every day.

TABLE 1

Primers for RT-qPCR

Gene		Primer sequences (5'–3')
GAPDH	Forward	GGAGTCCACTGGCGTCTTCA
	Reverse	ATTGCTGATGATCTTGAGGCTGTTG
Legumain	Forward	GCAGGTTCAAATGGCTGGTAT
	Reverse	GGAGTGGGATTGTCTTCAGAGT
Cdk2	Forward	GCCCTATTCCCTGGAGATTCTGAG
	Reverse	GGTACTGGCTTGGTCACATCCT
Cdk4	Forward	GAGCATGTAGACCAGGACCTAAGG
	Reverse	CCACCACTTGTCACCAGAATGTTC
Cdk6	Forward	GTGACCAGCAGCGGACAAAT
	Reverse	GCAGCCAACACTCCAGAGATC
CyclinD1	Forward	CCAGAGGCGGAGGAGAACAA
	Reverse	GAGGCGGTAGTAGGACAGGAAG
CyclinD3	Forward	GCTTACTGGATGCTGGAGGTATGT
	Reverse	AGCGTGGTCGGTGTAGATGC
Hoxa9	Forward	AATGAGAGCGGCGGAGACAA
	Reverse	TCGGTGAGGTTGAGCAGTCG
Hoxa10	Forward	CCTGGGCAATTCCAAAGGTGAAA
	Reverse	GGACGCTGCGGCTAATCTCTA
Meis1	Forward	CAGCAGTGAGCAAGGTGATGG
	Reverse	GGATGGTGAGTCCCGTGTCT
Pbx3	Forward	AGCATCACAGTGTCACAGGTATCC
	Reverse	GGCGAATTGGTCTGGTTGTTCTG
Oct4	Forward	CAAGCGATCAAGCAGCGACTATG
	Reverse	CAGAGTGGTGACGGAGACAGG
Sox2	Forward	CATGAACGGCTGGAGCAACG
	Reverse	CTGGAGTGGGAGGAAGAGGTAAC
Myc	Forward	CGTCCTCGGATTCTCTGCTCTC
	Reverse	GCTGCGTAGTTGTGCTGATGT

For the MTS assay, the Cell Proliferation Assay kit (Promega, Madison, WI) was used following the manufacturer's protocols. Briefly, cells were plated at a density of 1×10^4 cells/well in a 96-well plate. Two hours before each time point, 20 μL MTS reagent was added into each well. After incubation for 2 h, the absorbance at a wavelength of 490 nm was detected using a microplate reader.

Cell cycle analysis by PI staining

The detailed procedures of PI staining were described previously (Wang *et al.*, 2018b). Briefly, cells were collected and resuspended in 70% cold ethanol on ice for 1 h. Then, RNA was digested with 0.2 mg/mL RNase for 1 h. At last, PI was added at a final concentration of 10 μg/mL overnight at 4°C. Cells were analyzed on FACS. All experiments were repeated three times.

Cell apoptosis assay

Cells were collected and washed twice with annexin-V binding buffer. Then, cells were resuspended with annexin-V binding buffer. APC-conjugated annexin-V was added and incubated at room temperature for 15 min in the dark. Last, PI (500 μg/mL) was added. Flow cytometry was used to detect apoptotic cells. All experiments were repeated three times.

Cell migration assay

Transwell experiments were employed to evaluate cell migration. Cells were resuspended at a concentration of 1×10^6/mL in FBS free culture medium. An aliquot of 2×10^5 cells were added to the upper chamber (8.0-μm, Millicell), and 500 μL of 10% FBS medium was added to the lower chamber. After 24 h, membrane inserts were collected and non-invading cells on upper surface were removed. Then, the chamber membrane was fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. At least six fields of vision were randomly selected for cell counting under microscope. All experiments were repeated three times.

Colony-forming assay

Cells were collected and adjusted to a concentration of 1×10^5/mL in H4434 complete medium. Five hundred cells in 500 μL were seeded in a 24-well plate following the manufacturer's instructions. After seven days, colonies were counted under a microscope, and photos were taken by a high-content analysis system. Cell cluster with more than 50 cells was identified as one colony. All experiments were repeated three times.

G0 phase assay

Ki-67 staining was used for G0 phase analysis (Wang *et al.*, 2018a). Briefly, cells were harvested, fixed, and permeabilized by Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD, San Jose, CA). Then, PE-Cy7-conjugated Ki-67 was added and incubated for 30 min in the dark. Next, cells were washed twice and resuspended in PBS. Hoechst 33342 was added before analysis by flow cytometry. All experiments were repeated three times.

Statistical analysis

The results were expressed as mean ± SD. The analysis was done using GraphPad Prism 8.0 software. Comparisons between two groups were analyzed by unpaired Student's *t*-test. Statistically significant was accepted when the *p*-value was less than 0.05.

Results

Establishment of a THP1 cell line overexpressing legumain

THP1 cell line overexpressing legumain was constructed to study the role of legumain on leukemia cells. First, the legumain expressing vector was constructed. After digestion by *Xba* I and *Nhe* I, the PCR fragment was inserted into the vector pLV-EF1α-MCS-IRES-Bsd (Fig. 1(A)). The recombinant plasmids were verified by *Xba* I and *Nhe* I restriction endonuclease digestion. The lanes 1 and 2 were two recombinant plasmids. As expected, the bright bands between 2,000 bp and 1000 bp were observed (Fig. 1(B)). These plasmids were further verified by DNA sequencing, showing that their sequences were consistent with the sequence in NCBI (NM_005606.7). Hence, we successfully constructed the legumain expressing vectors.

Second, THP1 cells were infected with blank and recombinant lentiviruses. After Bsd selection, the blank lentivirus infected THP1 cell line was designated as the Vector group while the recombinant lentivirus infected one was designated as the Legumain group. The expression of

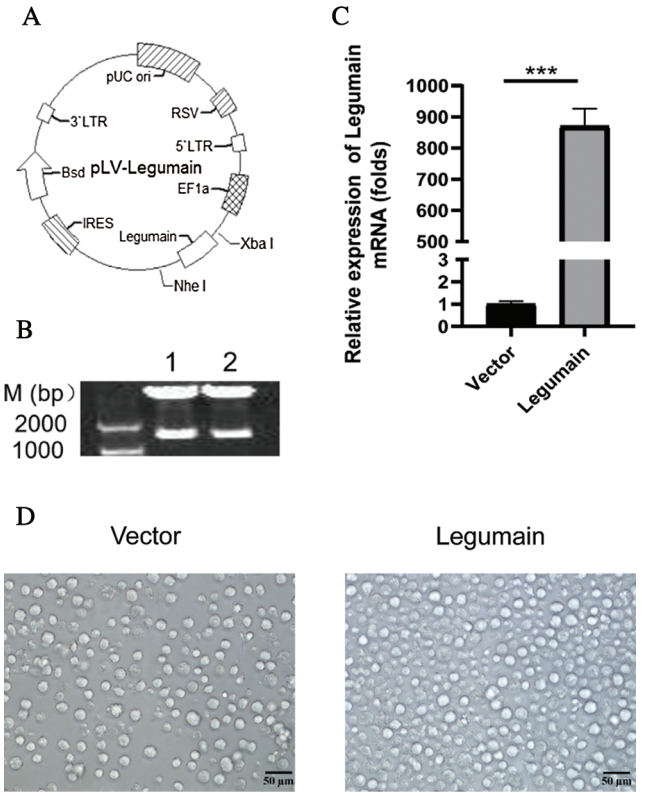

FIGURE 1. Establishment of an AML cell line overexpressing legumain.

(A) A diagram shows the structure of the recombinant vector overexpressing legumain. (B) After insertion and screening, recombinant plasmids were first verified by *Xba* I and *Nhe* I restriction endonuclease digestion. The typical electropherogram of two recombinant plasmids is shown. The DL 2000 DNA marker was loaded on the left. (C) Relative expression of legumain in Vector and Legumain groups was detected by qPCR. (D) Cell morphology of Vector and Legumain groups was observed under a microscope (Nikon). Scale bar: 50 μm. ***$p < 0.001$.

legumain was assessed by qPCR experiments showing that cells in the Legumain group expressed about 800-fold higher legumain than those in the Vector group (Fig. 1(C)). Meanwhile, the morphology of cells in the two groups showed little differences (Fig. 1(D)). The above results indicated that we successfully established the THP1 cells overexpressing legumain. In addition, the overexpression of legumain has little effect on cell morphology.

Effects of legumain on the proliferation and apoptosis of THP1 cells

We explored whether the high-level expression of legumain had an effect on the proliferation and apoptosis of leukemia cells. The results from both cell counting (Fig. 2(A)) and MTS (Fig. 2(B)) analyses demonstrated that the overexpression of legumain significantly enhanced cell proliferation when compared with the Vector group. PI staining was used for cell cycle analysis. The results indicated that more S phase and less G0/G1 phase were detected in cells in the Legumain group (Fig. 2(C)). The results from the qPCR of cell cycle-related factors showed that the overexpression of legumain significantly increased the expression of *Cdk6* and *Cyclin D1* (Fig. 2(D)). Annexin

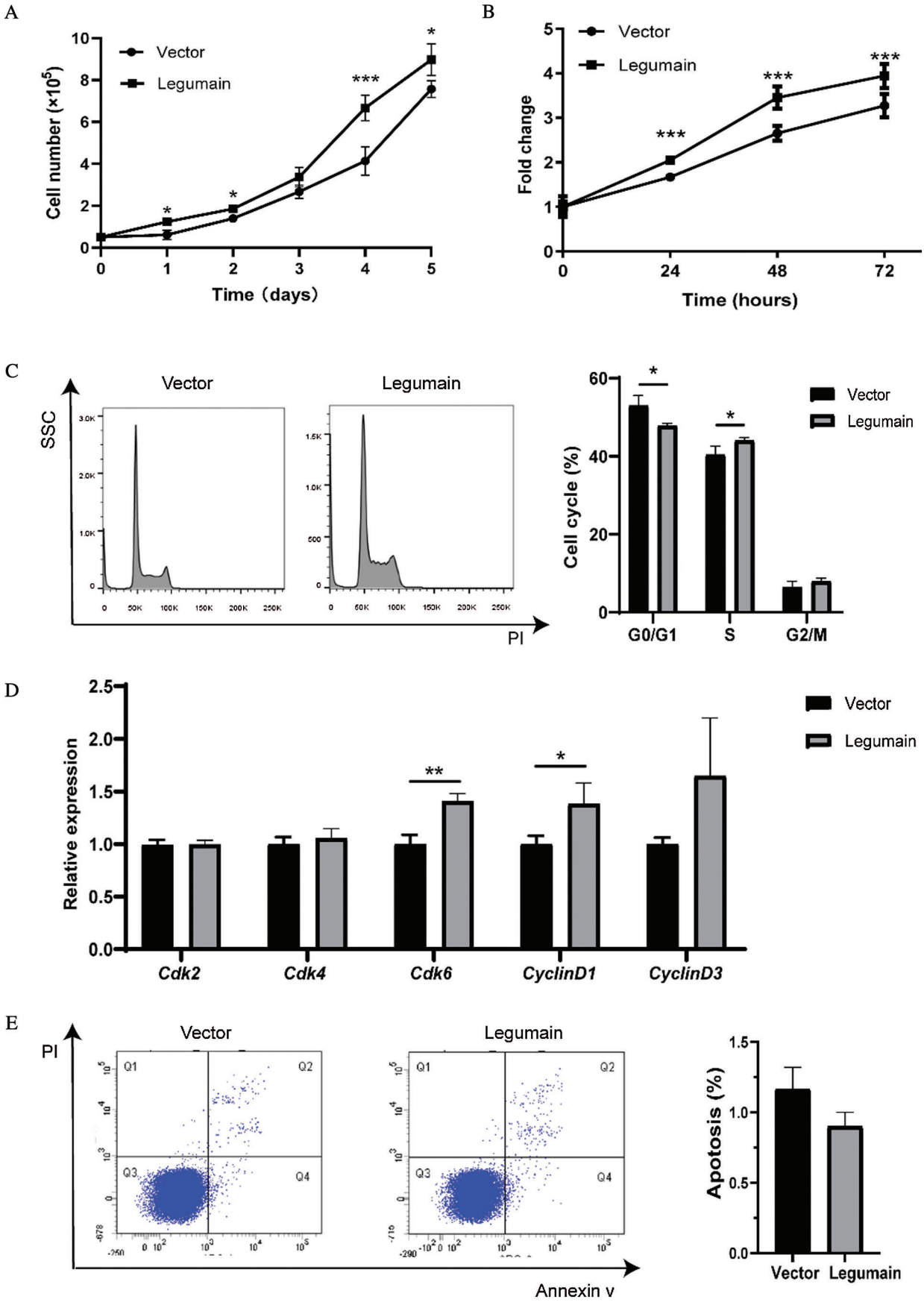

FIGURE 2. Effects of legumain on the proliferation and apoptosis of THP1 cells.
(A–B) The *in vitro* proliferation potential of cells in Vector and Legumain groups was assessed by cell counting (A) and the MTS method (B). (C) The cell cycle of THP1 cells in vector and Legumain groups was detected by PI staining, followed by FACS analysis. (D) The relative expression of cell cycle-related factors was detected by qPCR. (E) Cell apoptosis was detected by Annexin V-PI staining. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

V-PI analysis demonstrated that the overexpression of legumain had little effect on cell apoptosis (Fig. 2(E)).

The above results indicated that high-level expression of legumain promoted cell proliferation but had little effect on cell apoptosis in THP1 cells.

Effect of legumain on the migration of THP1 cells

We studied whether high-level expression of legumain affected the migration of leukemia cells by using Transwell experiments. Typical results are shown in Fig. 3(A). More migrated cells were detected in the Legumain group than the Vector group (Fig. 3(B)). These results suggested that the overexpression of legumain promoted cell migration potential in THP1 cells.

Effects of legumain on the stemness of THP1 cells

Finally, we studied whether the high-level expression of legumain affected the stemness of leukemia cells. The results of the colony-forming assay showed that fewer colonies were detected in the Legumain group than the Vector group

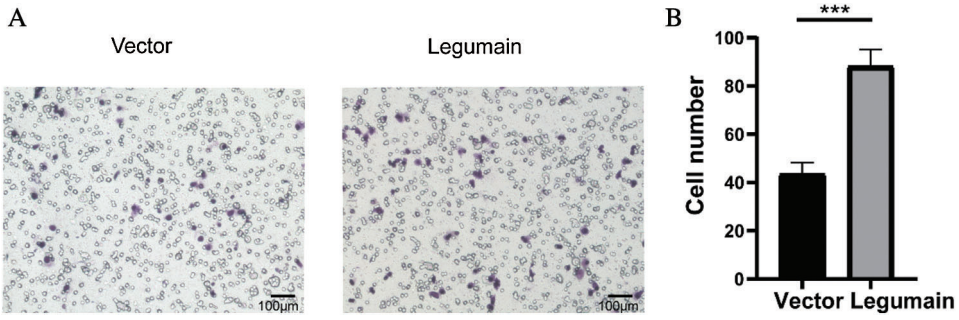

FIGURE 3. Effect of legumain on the migration of THP1 cells.
The migration potential of THP1 cells in Vector and Legumain groups was assessed by Transwell experiments. An aliquot of 2×10^5 cells was plated into the upper chamber and incubated for 24 h. The membranes were stained with 0.1% crystal violet. (A) The typical results under the microscope are shown. Scale bar: 100 μm. (B) The number of migrated cells each well is plotted. ***$p < 0.001$.

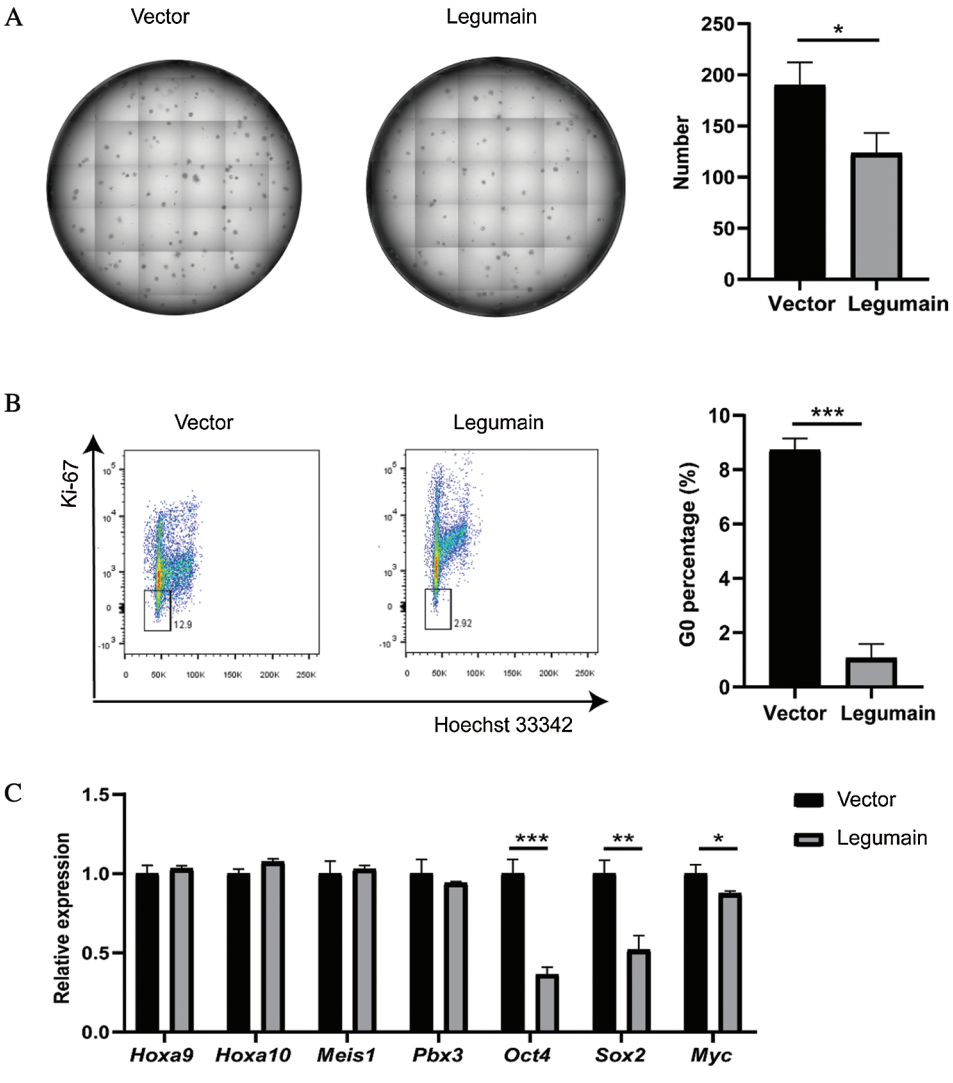

FIGURE 4. Effects of legumain on the stemness of THP1 cells.
(A) Cells in Vector and Legumain groups were cultured in H4434 for 7d. Typical photos were obtained by a high-content analysis system and are shown on the left. Colonies were counted under a microscope, and the number of colonies each well is plotted on the right. (B) Cells were subjected to Hoechst 33342 and Ki-67 staining before FACS analysis. Typical FACS results are shown, and the percentage of G0 phase cells is plotted. (C) The relative expression of classic genes involved in leukemia maintenance and stemness related transcription factors was detected by qPCR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(Fig. 4(A)). Then, the proportion of G0 phase cells was assessed by Hoechst 33342 and Ki-67 staining. The overexpression of legumain significantly decreased the proportion of G0 phase cells (Fig. 4(B)). The expression of related molecules was detected by qPCR (Fig. 4(C)). The overexpression of legumain had little effect on the expression of *Hoxa9*, *Hoxa10*, *Meis1*, and *Pbx3*, which had been suggested as key mediators in the transformation caused by MLL rearrangements (Ayton and Cleary, 2003; Li *et al.*, 2016; Orlovsky *et al.*, 2011; Wong *et al.*, 2007). However, the overexpression of legumain downregulated the expression of *Oct4*, *Sox2*, and *Myc*, which were important stemness markers in embryonic stem cells and cancer stem cells. These results suggested that the overexpression of legumain reduced the stemness of THP1 cells.

Discussion

Legumain plays roles under physiological conditions, especially in the formation of MHC class II (Manoury *et al.*, 2003). In recent years, more attention has been paid to the pathological effects of legumain. It has been demonstrated that legumain played key roles in neurodegenerative disease and neuroinflammation in cognitive impairment (Basurto-Islas *et al.*, 2013; Lian *et al.*, 2019). In solid tumors, tumor cells expressed a high-level of legumain when compared with normal tissues. The effects of legumain in solid tumors have been widely studied (Liu *et al.*, 2003). However, its effect on blood diseases has not been established. So, we aimed to investigate the role of legumain in leukemia cells in this study.

Legumain is expressed in different forms, i.e., cell surface, intracellular, and secretion forms (Fuchigami *et al.*, 2019). These forms may function through different mechanisms. Here, we inserted full-length cDNA of legumain into a lentiviral vector and successfully established THP1 cells overexpressing legumain by infection. A previous study demonstrated that unstimulated THP1 cells do not secrete legumain (Solberg *et al.*, 2015). Hence, we suggest that the majority of overexpressed legumain might be in intracellular and cell surface forms, whereas the secretion form cannot be excluded. Further work should be done to distinguish which form mainly contribute to the role of legumain in this model.

The effects of legumain on the proliferation and apoptosis of solid tumor cells have been well studied. In many cases, legumain promotes the proliferation of tumor cells (Liu *et al.*, 2003; Wang *et al.*, 2020). Furthermore, legumain promoted the proliferation of prostate cancer cells via the PI3K/AKT signaling pathway (Zhu *et al.*, 2016). However, the effect of legumain on apoptosis varied among cells. The knockdown of legumain resulted in the decrease of apoptosis in prostate cancer cells (Zhu *et al.*, 2016) but resulted in the increase of apoptosis in liver sinusoidal endothelial cells (Li *et al.*, 2019). In this report, we constructed THP1 cells overexpressing legumain to detect the effect of legumain on cell proliferation and apoptosis. The results demonstrate that the overexpression of legumain significantly promotes cell proliferation without affecting cell apoptosis in THP1 cells. The effects of legumain on cell apoptosis should be complicated and may be cell type-dependent. The exact mechanism should be further elucidated.

Cancer stem cells (CSCs) and leukemia stem cells (LSCs) play adverse pathological roles in the initiation, progression, and relapse of malignancies (Chavez-Gonzalez *et al.*, 2017). Although its pathological roles in solid tumors have been well studied, the effect of legumain on the CSCs has not been established. The *in vitro* colony-forming ability is one of the markers reflecting the self-renewal potential of leukemia cells. In this study, we discovered that the overexpression of legumain significantly decreased the colony-forming potential in THP1 cells. These results suggested that the overexpression of legumain reduced the self-renewal potential of leukemia cells. Furthermore, the overexpression of legumain in THP1 cells decreased the proportion of G0 phase cells. Moreover, the overexpression of legumain in THP1 cells decreased the expression of *Oot4*, *Sox2*, and *Myc*. These transcription factors are widely studied in embryonic stem cells and cancer stem cells (Vaddi *et al.*, 2019; Villodre *et al.*, 2019). In acute myeloid leukemia (AML), *Oct4* and *Sox2* were suggested as markers for LSCs (Picot *et al.*, 2017). It was also reported that overexpression of c-MYC initiated AML in a mouse model (Luo *et al.*, 2005). Hence, the overexpression of legumain may decrease the stemness of LSCs. The effects of legumain on the above-mentioned transcription factors have not been reported in other cancer cells. Whether the phenomenon occurs in other tumor cells and the related mechanism, should be further explored.

Legumain was suggested as a prognostic factor in certain solid tumors (Murthy *et al.*, 2005; Ohno *et al.*, 2013; Wang *et al.*, 2012). However, our results demonstrated the complicated effects of legumain in leukemia. On the one hand, it promoted the proliferation of leukemia cells. On the other hand, it decreased the stemness of leukemia cells. Whether legumain plays favorable or adverse roles in leukemia need further work to elucidate. In fact, the effect of legumain in leukemia may be more complicated since legumain can be secreted to the microenvironment. Besides acting as an intrinsic factor in malignant cells, legumain may also act as an extrinsic factor by affecting cells in the malignant microenvironment. Legumain is also expressed in macrophages (Liu *et al.*, 2014), which are important components of physiological and pathological microenvironments and play vital roles in the initiation and progression of solid tumors as well as leukemia (Chen *et al.*, 2015; Etzerodt *et al.*, 2020; Yang *et al.*, 2018a). Recent evidence showed that loss of legumain in macrophages promoted senescence of tumor cells (Shen *et al.*, 2019). Hence, both sides should be considered to unravel the role of legumain in leukemia.

Taken together, we demonstrate the complicated effects of legumain in leukemia cells. Legumain promotes the proliferation and migration of THP1 cells whereas decreases the stemness of THP1 cells.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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