Regenerative Therapy 27 (2024) 191–199

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Synergistic effect and molecular mechanism of nicotinamide and UM171 in *ex vivo* expansion of long-term hematopoietic stem cells

Yanni Cui [a], Yan Ren [a, b], Fanggang Ren [b, c], Yaofang Zhang [b, c], Hongwei Wang [a, b, c, d, *]

[a] The Second Clinical Medical College, Shanxi Medical University, Taiyuan, China
[b] Department of Hematology, The Second Hospital of Shanxi Medical University, Taiyuan, China
[c] Key Laboratory of Molecular Diagnosis and Treatment of Blood Diseases in Shanxi Province, Taiyuan, China
[d] Shanxi University of Chinese Medicine, Jinzhong, China

ARTICLE INFO

Article history:
Received 9 February 2024
Received in revised form
10 March 2024
Accepted 15 March 2024

Keywords:
Hematopoietic stem cells
Long-term hematopoietic stem cells
Nicotinamide
UM171
Umbilical cord blood
Self-renewal

ABSTRACT

Introduction: Several approaches to expand human hematopoietic stem cells (HSCs) have been reported, but the ability of these methods to expand long-term hematopoietic stem cells (LT-HSCs) remains to be improved, which limits the application of HSCs-based therapies.
Methods: CD34+ cells were purified from umbilical cord blood using MacsCD34 beads, and then cultured for 12 d in a serum-free medium. Flow cytometry was used to detect phenotype, cell cycle distribution, and apoptosis of the cultured cells. Colony-forming cell (CFC) assays can evaluate multi-lineage differentiation potential of HSCs. Real-time polymerase chain reaction was employed to detect the expression of genes related to self-renewal programs and antioxidant activity. DCFH-DA probes were used to evaluate intracellular production of reactive oxygen species (ROS). Determination of the effect of different culture conditions on the balance of cytokine by cytometric bead array.
Results: Here, we show a combination, Nicotinamide (NAM) combined with pyrimidoindole derivative UM171, can massively expand LT-HSCs *ex vivo*, and the expanded cells maintained the capability of self-renewal and multilineage differentiation. Additionally, our data indicated that UM171 promoted self-renewal of HSCs by inducing HSCs entry into the cell cycle and activating Notch and Wnt pathways, but the infinite occurrence of this process may lead to mitochondrial metabolism disorder and differentiation of HSCs. NAM kept HSCs in their primitive and dormant states by reducing intracellular ROS levels and upregulating the expression of stemness related genes, so we believed that NAM can act as a brake to control the above process.
Conclusions: The discovery of the synergistic effect of NAM and UM171 for expanding LT-HSCs provides a new strategy in solving the clinical issue of limited numbers of HSCs.

1. Introduction

Development and maintenance of the hematopoietic system rely on hematopoietic stem cells (HSCs) which are characterized by the capability of self-renewal and the ability to give rise to all types of blood cells. HSCs in the graft will generate healthy cells to cure or replace the impaired cells in patients with hematologic malignancies or immunodeficiencies. Umbilical cord blood (UCB) is a suitable alternative source of HSCs, however, transplantation of the limited number of HSCs that are present in single UCB units is associated with delayed engraftment and increased graft failure [1]. Expansion of HSCs *ex vivo* is an effective way to overcome this problem. Unfortunately, HSCs cultured *in vitro* are prone to differentiation, leading to loss of stemness and impaired multi-lineage differentiation potential [2,3]. According to the reconstruction kinetics of HSCs after primary and secondary recipient transplantation, HSCs can be divided into long-term hematopoietic stem cells (LT-HSCs) and short-term ones (ST-HSCs). LT-HSCs are a group of primitive cells in the hematopoietic system, which maintained donor chimerism for at least 16 weeks, while ST-HSCs did not provide lasting chimerism for any lineage after transplantation [4,5]. Although freshly isolated CD34+ cells contain a population of LT-HSCs, most CD34+ cells are lineage restricted progenitor cells and do not have long-term engraftment potential [6]. Therefore, the current priority for UCB

* Corresponding author. Department of Hematology, The Second Hospital of Shanxi Medical University, 382 Wuyi Road, Taiyuan 030001, China.
E-mail address: wanghw68@hotmail.com (H. Wang).
Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2024.03.011

Abbreviations

BFU-E	burst forming units-erythroid
CAT	catalase
CFC	colony-forming cell
CFU-GEMM	colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	colony forming units-granulocyte, macrophage;
FLT-3L	Flt-3 ligand
HSCs	hematopoietic stem cells
HSPCs	hematopoietic stem and progenitor cells
IFN-γ	interferon-γ
IL	interleukin
LT-HSCs	long-term hematopoietic stem cells
MFI	mean fluorescence intensity
NAD+	nicotinamide adenine dinucleotide
NAM	nicotinamide
ROS	reactive oxygen species
SCF	stem cell factor
SIRT1	Sirtuin 1
SOD-1	superoxide dismutase-1
ST-HSCs	short-term hematopoietic stem cells
TGF-β	transforming groeth factor-β
TNC	total nucleated cells
TNF-α	tumor necrosis factor-α
TPO	thrombopoietin
UCB	umbilical cord blood

transplantation is to develop efficient methods to expand LT-HSCs *ex vivo* and thus improve transplantation outcomes.

At present, a variety of cytokines showed the ability of expanding hematopoietic stem and progenitor cells (HSPCs) *ex vivo*, including stem cell factor (SCF), FMS-like tyrosine kinase-3 ligand (FLT3-L), thrombopoietin (TPO) et al. [7–9]. And small molecules are emerging as valuable tools for regulating stem cell fates. Recent studies have shown that StemRegenin 1(SR1), Nicotinamide (NAM) and pyrimidoindole derivative UM171 displayed excellent effect on HSC expansion [10–12]. NAM, a water-soluble vitamin B-3, is a well-established inhibitor of enzymes that rely on nicotinamide adenine dinucleotide (NAD+) activity, such as Sirtuin 1 (SIRT1) [13]. UM171 is a newly synthesized UM729 analogue, which has a nearly 20 fold increased ability to promote the expansion of CD34+CD45RA-cells compared to UM729. However, the effects of the above substances on the expansion of LT-HSCs and the molecular mechanism of action remains unclear.

In this study, we investigated the effect of the combination of NAM and UM171 on HSC expansion *ex vivo*. In humans, CD34+CD38-cells are classic HSPCs, and HSCs can be functionally divided into ST-HSCs (CD34+CD38-CD45RA-CD49f+CD90-) and LT-HSCs (CD34+CD38-CD45RA-CD49f+CD90+) [14–20]. We found that NAM+UM171 exhibited superior activity in expanding phenotype defined LT-HSCs proportionally and quantitatively, the expansion folds of LT-HSCs reached 753.16 ± 83.02. Compared with Dimethyl sulfoxide (DMSO), NAM significantly increased the proportion of HSPCs, ST-HSCs and LT-HSCs, as well as dramatically upregulated the expression of stemness-related genes and reduced the production of reactive oxygen species (ROS), which were beneficial for maintaining stemness and inhibiting differentiation of HSCs. After UM171 and NAM combined treatment, the proportion of HSPCs, ST-HSCs and LT-HSCs further increased, and downstream genes of Notch and Wnt pathways were selectively activated. In conclusion, the antioxidant properties of NAM can delay differentiation, while UM171 can promote self-renewal by regulating the stem cell pathways, and the combination of them is beneficial for the maintenance and expansion of HSCs *ex vivo*. All results showed that the NAM+UM171 combination was a hopeful selection for massive expansion of LT-HSCs.

2. Materials and methods

2.1. Sample preparation and CD34+ cells isolation from human UCB

Samples were collected from consenting donors according to the ethical procedures approved by the second hospital of Shanxi Medical University (Shanxi, China). Cord blood mononuclear cells (MNCs) were isolated with hydroxyethyl starch (HES) and Ficoll (Tianjin Haoyang. TBD), and then wash and resuspend them in column buffer (PBS and 0.5% bovine serum albumin. Gibco). Isolation of CD34+ cells from MNCs using CD34 MicroBeads and LS Columns (Miltenyi Biotec) associated with a magnetic field in the last step.

2.2. Cell culture

CD34+ cells were seeded at 5×10^4/mL in serum-free medium, which was composed of StemSpan Serum-free Expansion Medium (SFEM, StemCell Technologies), supplemented with 10 ng/ml human stem cell factor (SCF, Miltenyi biotec), 100 ng/ml thrombopoietin (TPO, Miltenyi biotec), 1% Penicillin-Streptomycin-Glutamine (Gibco). Different concentrations of NAM (Solarbio) or UM171 (Sigma-Aldrich) at 35 nM were added according to grouping requirements (The concentration of UM171 refers to the previous data from our laboratory) [21]. Incubate cells in a humidified incubator at 37 °C, 5% CO2 and 5% O2 for 12 days. Small molecule NAM and UM171 were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and stored as stock solutions, stock solutions were diluted to working solutions at the desired concentration by serum-free medium.

2.3. Phenotypic analysis

Total expanded cells were collected, and the live cells were counted using AO/PI (Thermo Scientific) and an automated cell counter (Thermo Scientific) after culture. Cells were stained in phosphate buffered saline (PBS, Gibco) supplemented with the following antibody and fluorophore combinations for 30 min at 4 °C: ECD-labeled anti-human CD34 (BECKMAN; IM2709U), FITC-labeled anti-human CD38 (BECKMAN; A07778), Pacific Blue-labeled anti-human CD45RA (BECKMAN; A82946), APC-CY7-labeled anti-human CD49f (Biolegend; 313628) and PerCP-CY5-labeled anti-human CD90 (BECKMAN; IM3703). After the cleaning step, the stained cells were analyzed by Navios (BECKMAN COULTER) flow cytometry. For each sample, at least 30,000 events were recorded. Isotype controls were also prepared for every experiment.

2.4. Apoptosis

Collect cells and wash twice with pre-cooled PBS. Cells were stained for 15 min at RT with PE-Annexin V Apoptosis Detection Kit (BD, Biosciences). Samples were then immediately analyzed by Navios flow cytometer within 1 h. Results are expressed as

percentage of live cells, early apoptotic cells, late apoptotic cells and necrotic cells compared to the whole cells.

2.5. Cell cycle

Cells were washed with precooled PBS, resuspend in 70% ethanol at 4 °C for 2 h or more, and then washed with PBS, incubated with 1.0 ml propidium staining solution (Bioss) for 30 min at 37 °C in the dark. A minimum of 40,000 gated events was collected for each sample.

2.6. Colony forming cell assay (CFC)

According to the cell count results, adjust the concentration of CD34+ cells of each group to 1×10^5/mL, add 50 μL cells to 450 μL Iscove's Modified Dulbecco's Medium (IMDM, Gibco) for mixing, and then add 150 μL cells to 1.5 mL MethodCult™ medium (StemCell Technologies) for fully shaking and mixing. Connect a 1.6 mm needle to a 2 mL disposable sterile syringe and distribute the MethodCultTM mixture containing cells into a 35 mm culture dish (Corning), so that the culture medium is evenly distributed on the surface of each culture dish. The cells were cultured in 5% CO2 incubator at 37 °C for 14 days, and culture dishes were visually scored for CFU-GM (colony-forming unit granulocyte/macrophage), BFU-E (burst-forming unit erythrocyte) and CFU-GEMM (colony-forming unit granulocyte/erythrocyte/macrophage/megakaryocyte).

2.7. ROS assay

The cultured cells were resuspended at a density of 5×10^5 to 1×10^6 cells/mL, and dilute the DCFH-DA probe with serum free medium according to the manufacturer's instructions (Beyotime Biotechnology). Resuspend cells with diluted DCFH-DA and incubate for 20 min in a 37 °C incubator. Mix it upside down every 3–5 min to ensure full contact between the probe and the cell. Wash the cells with serum free medium three times to fully remove DCFH-DA that has not entered the cells. The fluorescence intensity was measured within 2 h by Navios flow cytometer. The median level of MFI (mean fluorescence intensity) of ROS was obtained in software Kaluza (Beckman) of each sample to the same measuring cell number.

2.8. Cytokine assay

This assay was conducted according to the Instruction Manual of BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (Catalog No. 560484). The fresh cytokine standards were prepare to run with each experiment. Add 50 μL of sample and 50 μL of the Human Th1/Th2/Th17 PE Detection Reagent to the sample tubes and incubate the assay tubes for 3 h at room temperature, protected from light. Analyze dates using FCAP Array software.

2.9. Real-time PCR (RT-PCR)

Total RNA was extracted by Trizol method. For cDNA synthesis, total RNA was reverse-transcribed with an cDNA synthesis kit (TaKaRa, Japan). PCR was performed using a SYBR Premix Ex TaqTMII (TaKaRa, Japan) and the CFX96 real-time PCR detection system (BioRad, CA). Each reaction was repeated at least three times to demonstrate reproducibility, and data were analyzed using the CFX96 Real-TimeSystem. Normalized values were obtained by subtracting the threshold cycle (Ct) of β-actin from the Ct values of the target genes, yielding △Ct values, and the △△Ct formula was used as an indication of the relative transcriptional level. The primer sequences used were:

SIRT1 forward (5′- CAAACTTTGCTGTAACCCTGT-3′) and reverse (5′-CAGCCACTGA AGTTCTTTCAT-3′);
CAT forward (5′- TGGGATCTCGTTGGAAATAACAC-3′) and reverse (5′-TCAGGACGTAGGCTCCAGAAG-3′);
SOD1 forward (5′-GGTGGGCCAAAGGATGAAGAG-3′) and reverse (5′-CCACAAGCCAAACGACTTCC-3′);
AXIN2 forward (5′-GCAACTCAGTAACAGCCCGA-3′) and reverse (5′-CTCCTCTCTTTTACAGCAGGGC-3′);
MMP7 forward (5′-GTCTCTGGACGGCAGCTATG-3′) and reverse (5′-GATAGTCCTGAGCCTGTTCCC-3′);
HES1 forward (5′-GTGTCAACACGACACCGGAT-3′) and reverse (5′-GGAATGCCGCGAGCTATCTT-3′);
HES3 forward (5′-GATTTCCAAGCCGCTGATGG-3′) and reverse (5′-TTCCGGATCTGGTGCGAGTA-3′);
HEY1 forward (5′-TGCGGATTGAGCTAGTGCAT-3′) and reverse (5′-AAGTAACCTTGGTCTCCCGT-3′);
BMI1 forward (5′-CGTGTATTGTTCGTTACCTGGA-3′) and reverse (5′-TTCAGTAGTGGTCTGGTCTTGT-3′);
HOXB4 forward (5′-CGTGAGCACGGTAAACCCC-3′) and reverse (5′-CGAGCGGATCTTGGTGTTG-3′);
GATA2 forward (5′-ACTGACGGAGAGCATGAAGAT-3′) and reverse (5′-CCGGCACATAGGAGGGGTA-3′);
β-actin forward (5′-AGAGCTACGAGCTGCCTGAC-3′) and reverse (5′-AGCACTGTGTTGGCGTACAG-3′);

2.10. Statistical analysis

The significant differences between each group were analyzed using SPSS 22.0 statistical software for all experimental data. The comparison was analyzed between two groups with an independent sample *t*-test and among three groups with single-factor analysis of variance (ANOVA). The values were plotted as the mean ± standard deviation. Probability values $P < 0.05$ were considered statistically significant.

3. Results

3.1. Identification of optimal working concentration of NAM

The normal plasma concentration of NAM is around 5 μM [22]. High dosage of NAM is often required in clinical treatment, and the concentration in serum could reach the millimolar range. Researchers have an inconsistent understanding of the specific concentration at which NAM exerts its effects [22,23]. To investigate the optimal working concentration of NAM under our laboratory conditions, UCB CD34+ cells were incubated in serum-free medium with different concentrations of NAM for 12 days. And equivalent DMSO was used as a control. Flow cytometry was performed to analyze the percentage of subpopulations based on the expression of cell surface markers: CD34, CD38, CD45RA, CD49f and CD90, which were always used to define a more primitive subpopulation of HSCs.

The concentration gradient of NAM was set to 0 mM, 2 mM, 4 mM, 6 mM, 8 mM, 10 mM and 12 mM. When the concentration of NAM was 6 mM, 8 mM, and 10 mM, the proportion of HSPCs (CD34+CD38-cells) and LT-HSCs (CD34+CD38-CD45RA-CD49f+CD90+ cells) were significantly higher than that of the control group (Fig. 1A and B), and the expansion folds of HSPCs and LT-HSCs in the 6 mM group were higher than those in the other groups (Supplementary Table 1). This may be due to a dose-

dependent increase in the proportion of dead cells when the concentration was higher than 6 mM (Fig. 1C). Thus, our group identified 6 mM (used in subsequent experiments) as the optimal working concentration of NAM to promote the expansion of HSCs.

3.2. NAM and UM171 coordinately enhanced expansion of phenotype-defined LT-HSCs ex vivo

HSCs are a group of heterogeneous cells that include multipotent progenitors, ST-HSCs and LT-HSCs, in which the multipotent progenitors and ST-HSCs are able to provide early and transient hematopoietic recovery, LT-HSCs can maintain multi-lineage hematopoiesis for more than 4 months after transplantation to the recipient, whereas its characteristic is delayed implantation [24]. Thus, we compared the percentage and expansion folds of HSPCs, ST-HSCs and LT-HSCs through flow cytometry analysis to determine the expansion efficiency of different groups (DMSO, NAM, UM171, NAM+UM171).

The results showed that the percentage and expansion folds of HSPCs, ST-HSCs (CD34+CD38-CD45RA-CD49f+CD90-cells) and LT-HSCs were significantly increased in the NAM and UM171 groups compared with DMSO group (Fig. 2A–D, Supplementary Table 2). This is consistent with the change of the total nucleated cells (TNC) expansion folds (Fig. 2E). While UM171 treatment significantly increased the number of LT-HSCs rather than percentage compared with NAM (Fig. 2C). The joint intervention of NAM and UM171 increased quantity and proportion of HSPCs and ST-HSCs, which is more efficient than either of them treated alone (Fig. 2A and B). Notably, the expansion folds of LT-HSCs in NAM+UM171 group can reach 753.16 ± 83.02, which was approximately 65 times higher than that in the DMSO group, far higher than 8 and 16 times when treated with NAM and UM171 alone (Supplementary Table 2). This cooperation effect could significantly improve the problem of insufficient expansion of LT-HSCs. In addition, we found that the effect of NAM+UM171 treatment on maintaining stem cell characteristics under normoxic conditions is not as effective as under 5% O2 hypoxia conditions (Supplementary Fig. 1). These results indicated that UM171 had a better effect on the number of HSCs subsets than NAM during *ex vivo* expansion. Furthermore, NAM and UM171 had a significant synergistic effect on the expansion of LT-HSCs.

3.3. NAM treatment imposed negative impact on cell apoptosis, and UM171 can reverse this phenomenon

HSCs aging, which is accompanied by reduced self-renewal ability, impaired homing, myeloid-biased differentiation and other defects in hematopoietic reconstitution function, and in all transplantation studies in which young HSCs were competed against aged HSCs, without exception the young stem cells are functionally superior [25,26]. Senescent cells display an enlarged morphology coupled with a proliferation arrest [27]. In order to analyze the effects of different culture conditions on HSCs aging, we conducted related experiments.

Compared with DMSO, NAM treatment has no significant effect on the cycle distribution of HSCs (Fig. 3A,B,C). However, UM171 treated cells exhibited a decrease of the G0/G1 phase and an increase of the S/G2/M phase compared with DMSO, which was more pronounced than the NAM+UM171 group (Fig. 3C). This indicates that the combination with UM171 can significantly improve NAM's inability to adequately induce HSCs entry into the cell cycle. Besides, the cell diameter of the NAM group is much smaller than that of the DMSO and UM171 groups, suggesting that NAM was more conducive to maintaining the morphology of HSCs (Fig. 3D). To further analyze the mechanism of NAM and UM171 in HSC expansion, cell apoptosis assay was performed. The results showed that NAM treatment resulted in an increase in early apoptosis cells, while it did not impose any significant impact on late apoptotic cells and necrotic cells (Fig. 3E and F). Compared with the DMSO and NAM groups, the proportion of early apoptotic cells in the UM171 and NAM+UM171 groups significantly decreased, while the proportion of surviving cells correspondingly increased (Fig. 3E and F). The outcomes were consistent with the total nucleated cells (TNC) expansion folds, indicating that UM171 can reverse the negative effect of NAM treatment on the apoptosis of HSCs. In summary, during *ex vivo* culture, NAM+UM171 can delay the aging of HSCs, promote cell survival and self-renewal.

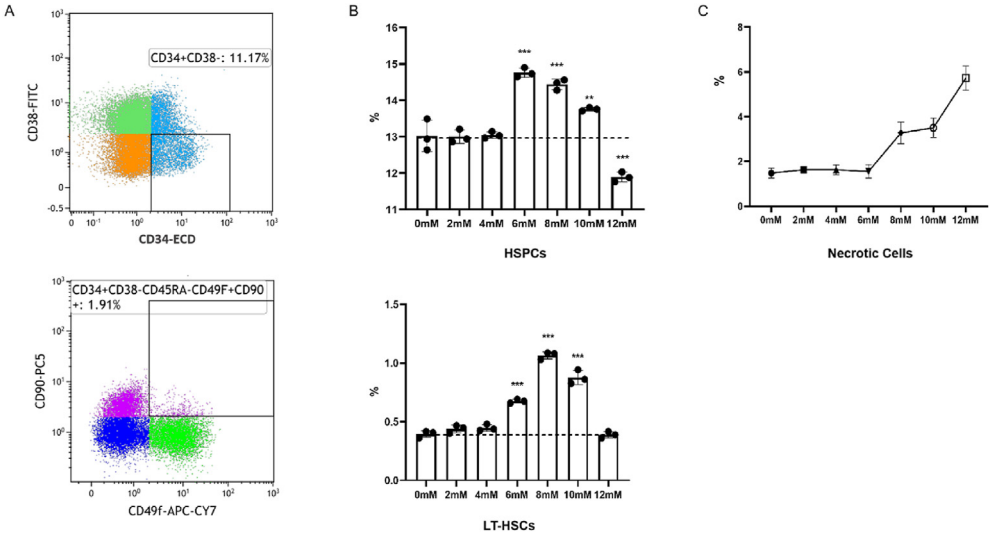

Fig. 1. Identification of the optimal working concentration of NAM for *ex vivo* expansion of human UCB CD34+ cells. (A) Representative FACS profiles of HSPCs (CD34+CD38-cells) and LT-HSCs (CD34+CD38-CD45RA-CD49f+CD90+ cells) subpopulations. (B) Percentage of HSPCs and LT-HSC subpopulations in cells cultured with DMSO or different concentrations of NAM (2 mM, 4 mM, 6 mM, 8 mM, 10 mM, 12 mM) for 12 days ($n = 3$). (C) The proportion of necrotic cells *ex vivo* cultured cells of each group as described in (C) ($n = 3$). The data were drawn from three independent experiments and the results were expressed as mean ± SD. Compared with control group unless specified. $^{**}P < 0.01$, $^{***}P < 0.001$ and ns = not significant by two-tailed unpaired Students'*t*-test.

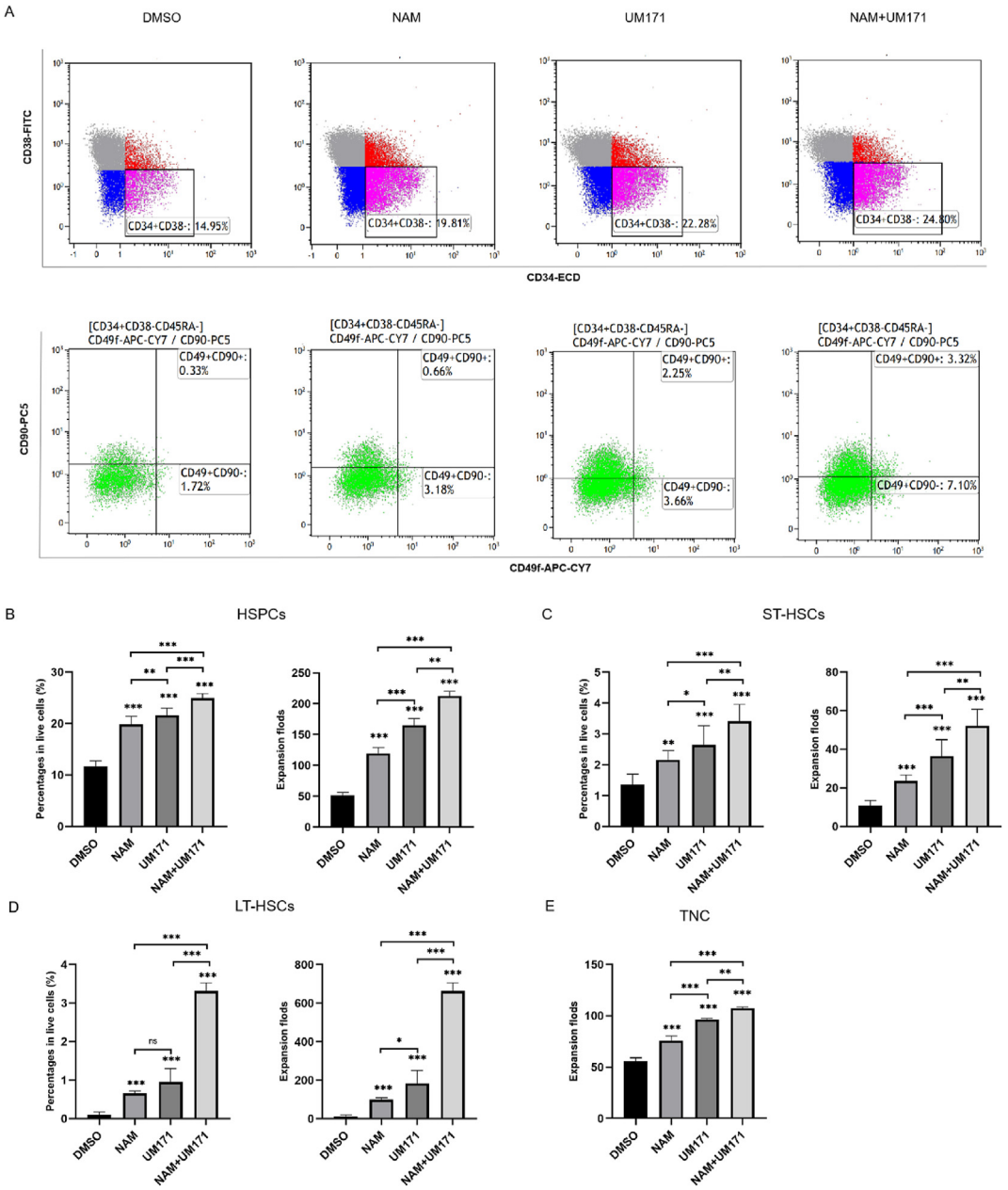

Fig. 2. CO-incubation with NAM and UM171 promoted expansion of UCB LT-HSCs *ex vivo*. (A) Representative FACS profiles of HSPCs (CD34+CD38-cells), ST-HSCs (CD34+CD38-CD45RA-CD49f+CD90-cells) and LT-HSCs (CD34+CD38-CD45RA-CD49f+CD90+cells) subpopulations. The percentages in live cells and the expansion folds of HSPCs (CD34+CD38-cells) (B), ST-HSCs (CD34+CD38-CD45RA-CD49f+CD90-cells) (C), LT-HSCs (CD34+CD38-CD45RA-CD49f+CD90+cells) (D) subpopulations and TNC (E) after a serum-free 12 days culture with DMSO, NAM, UM171, NAM+UM171 (n = 4). The data were drawn from four independent experiments and the results were expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and ns = not significant by two-tailed unpaired Students' *t*-test.

3.4. NAM+UM171 sustained stemness and multi-lineage differentiation potential of HSCs

Hematopoietic colony-forming cell (CFC) assays represent a classical tool for quantifying and evaluating multi-lineage differentiation potential of HSCs [28]. Thus, we evaluated the function of each group of cells through CFC assays. CD34+ cells isolated from UCB were divided into different groups for culture, and then seeded into MethodCultTM medium. After 14 days, colony formation was observed under an inverted microscope including Burst-forming unit-erythroid (BFU-E), Colony-forming unit-granulocyte, macrophage (CFU-GM) and Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) [29].

There was no difference in the distribution or morphology of colonies among all groups. It was shown that compared with DMSO, BFU-E and CFU-GEMM produced by NAM treated cells increased by 1.72- and 2.25-fold, the CFU-GM of UM171 group increased by 1.80 times and GEMM increased by 2.50 times (Fig. 4A and B). The numbers of total colonies, CFU-GM, BFU-E and CFU-GEMM in NAM+UM171 group increased by 1.88-, 1.99-, 1.58- and 4.63-fold, respectively (Fig. 4A and B). The CFU-GEMM, representing the more primitive and multipotent progenitor cells, were significantly higher in NAM+UM171 group than that in NAM or UM171 group. These results indicated that NAM+UM171 can better maintain the multipotentiality and long-term repopulating activity of HSCs during *ex vivo* culture.

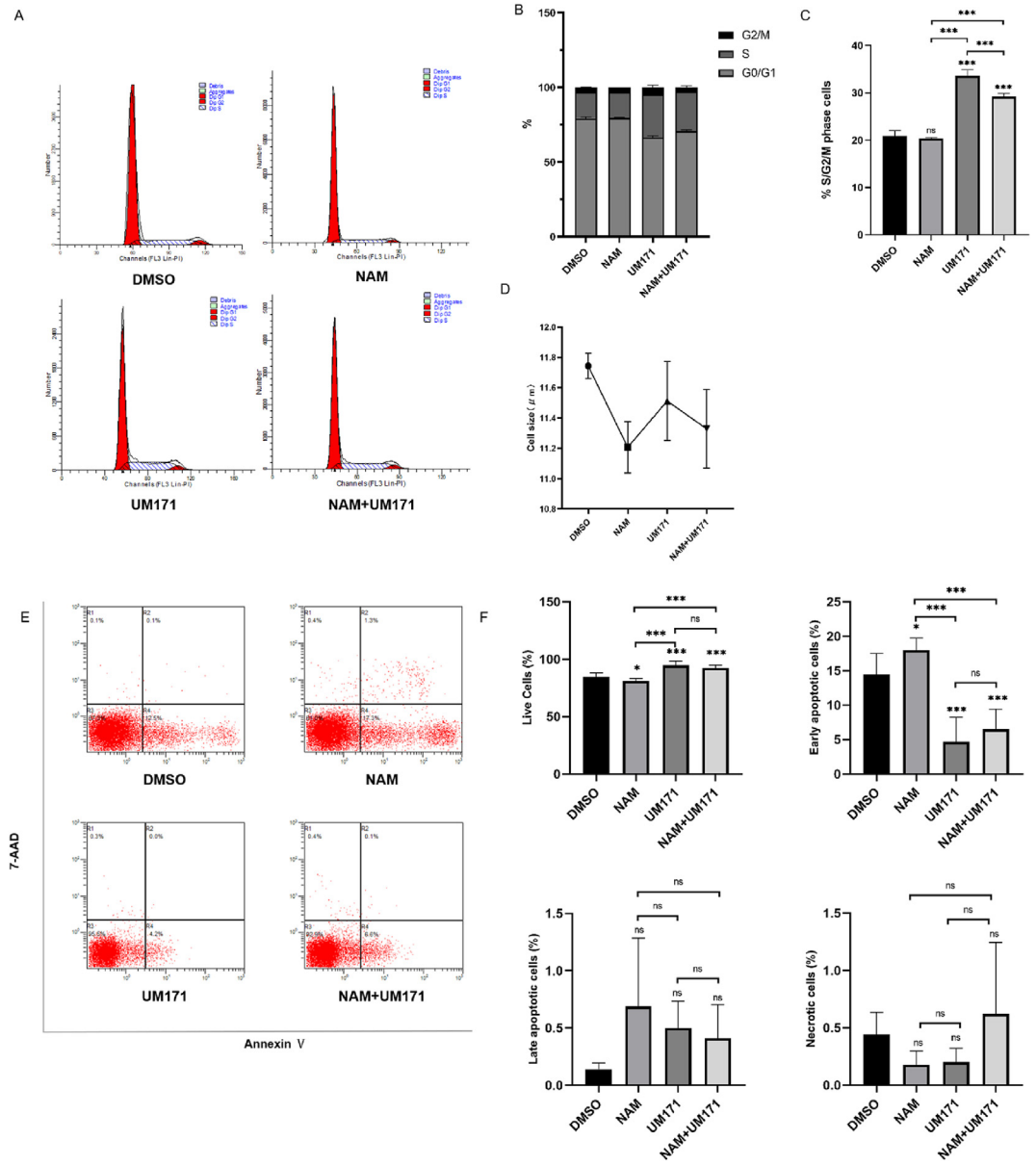

Fig. 3. The addition of UM171 not only reversed the effect of NAM on cell apoptosis, and also showed high proliferation ability and more primitive cell morphology in cells treated with NAM+UM171. The representative diagram of flow cytometry analysis (A) and the cell cycle distribution (B) of G0/G1, S and G2/M phase, and the statistical values of the S/G2/M phase (C) ($n = 6$). (D) Average cell size of live cells cultured *ex vivo* for 12 days ($n = 6$). (F)Statistics of early apoptotic cells, late apoptotic cells, and necrotic cells after treatment with DMSO, NAM, UM171 and NAM+UM171, and the representative diagram of flow cytometry analysis (E) ($n = 6$). The data were drawn from six independent experiments and the results were expressed as mean ± SD. ***$P < 0.001$ and ns = not significant by two-tailed unpaired Students' *t*-test.

3.5. NAM+UM171 induced LT-HSCs self-renewal by activating Wnt and notch signaling and enhancing antioxidant activity

Self-renewal of HSCs requires stemness maintenance and cell proliferation. Several studies have demonstrated that HSCs generate energy mainly through anaerobic glycolysis by limiting the production of ROS to maintain their stemness and quiescence [30,31]. To test whether *ex vivo* expanded cells exhibit self-renewal properties, we analyzed the expression of genes related to self-renewal programs and antioxidant activity.

First, we measured ROS levels in HSCs cultured in different conditions, and intracellular ROS levels were indicated by mean fluorescence intensity (MFI). NAM, UM171 or NAM+UM171 treatment significantly decreased the ROS levels in cytoplasm compared with that of the DMSO group (Fig. 5A). Noticeably, the intracellular ROS levels in UM171 and NAM+UM171 groups were higher than NAM group (Fig. 5A). Consistent with this, we observed that NAM significantly up-regulated the expression level of superoxide dismutase-1 (SOD-1), UM171 could up-regulate the catalase (CAT) but has no effect on SOD-1 (Fig. 5B). In addition, the addition of UM171 increased the transcription level of SIRT1 and weakened the effect of NAM as a SIRT1 inhibitor (Fig. 5C). Furthermore, the stemness-related gene, GATA2, was found to be up-regulated in groups other than DMSO, while compared with the DMSO group, the expression of BMI-1 was downregulated in the NAM+UM171 group (Fig. 5D). In addition, we found that the downstream genes of the Notch pathway (HEY1, HES1, and HES3) and Wnt pathway (MMP7) in the NAM group were significantly lower than those in the DMSO group (Fig. 5E and F). Interestingly, Notch and Wnt pathways were significantly activated in the NAM+UM171 group,

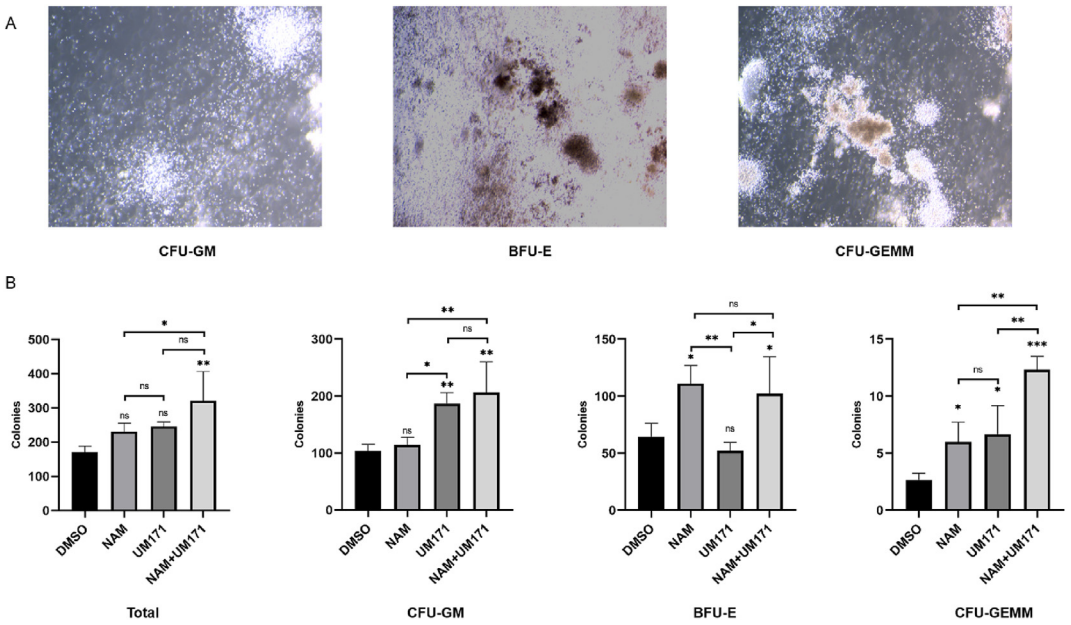

Fig. 4. NAM+UM171 sustained self-maintenance and multi-lineage differentiation potential of hematopoietic stem cells. (A) Typical images of representative morphologies of various type of colonies as indicated. (B) Colony formation assay showing the number of various types of cell colonies as indicated. Cells treated with DMSO, NAM, UM171, NAM+UM171 were cultured with H4434 methylcellulose for 14 days. The number of Burst-forming unit-erythroid (BFU-E), Colony-forming unit-granulocyte, macrophage (CFU-GM) and Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) were scored ($n = 4$). The data were drawn from four independent experiments and the results were expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ns = not significant by two-tailed unpaired Students' *t*-test.

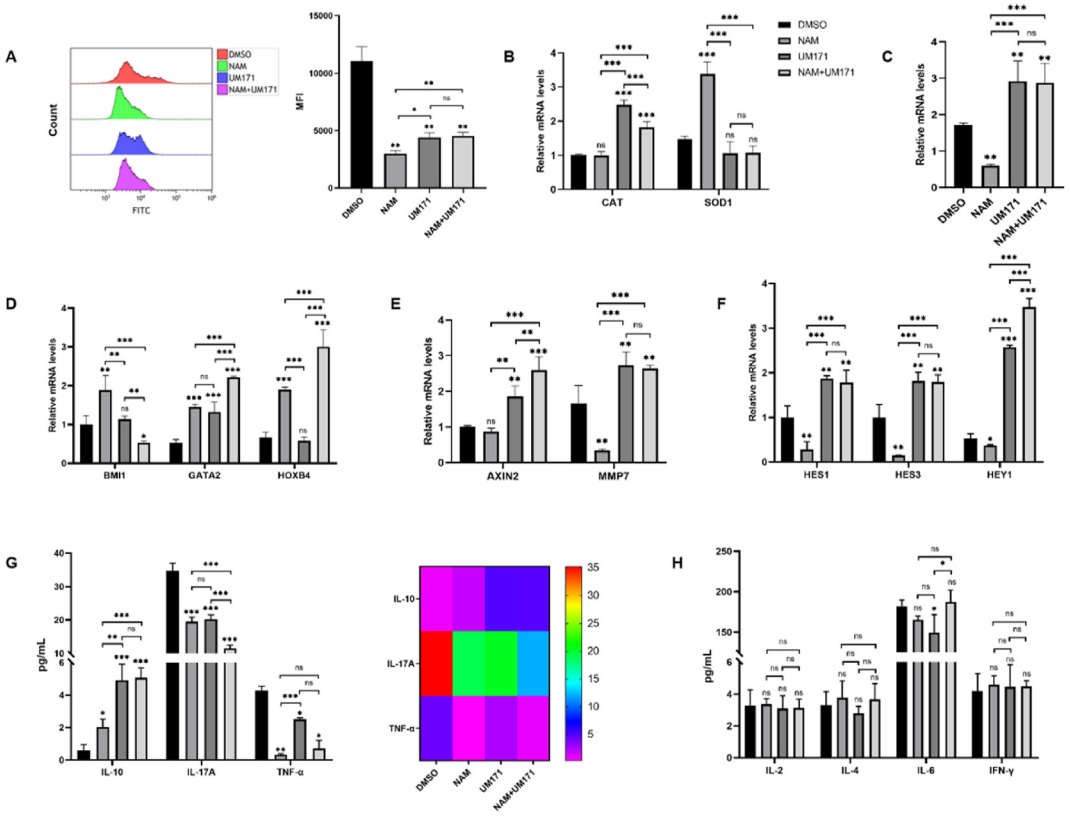

Fig. 5. Treatment by NAM+UM171 modifies the expression of HSC-related genes and the secretion of inflammatory factors. (A) Intracellular ROS levels (n = 4). Expression of antioxidant enzyme genes(B), SIRT1(C), stemness-related genes(D), Wnt(E) and Notch signaling genes(F) in DMSO, NAM, UM171 and NAM+UM171 cultured HSCs by real-time PCR(n = 4). (G-H) The levels of seven inflammatory cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, IFN-γ) in the cell supernatant ($n = 4$). The data were drawn from four independent experiments and the results were expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ns = not significant by two-tailed unpaired Students' *t*-.test.

and the expression of HEY1 and AXIN2 were significantly higher than those in the UM171 group (Fig. 5E and F). Further, the levels of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17A (IL-17A), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) in the supernatant of cultured cells were detected, and the results showed that the level of anti-inflammatory factor IL-10 was increased, and the levels of pro-inflammatory factors IL-17A and TNF-α were decreased in NAM+UM171 group, while there was no significant change in other cytokines (Fig. 5G and H). We hypothesized that the combination of NAM and UM171 could better balance secretion of pro-inflammatory and anti-inflammatory cytokines.

4. Discussion

HSCs are defined by its capacity to continuingly (reflecting the self-renewal feature) give rise to all the blood cell types (reflecting the differentiation potential) [32]. Human UCB cells have exhibited promising potential as an alternative source for HSCs, but the number of HSCs from a single UCB unit is not enough for adult transplantation. Therefore, the study of *in vitro* expansion conditions for HSCs has great clinical significance. The niche plays a crucial role in regulating HSCs fate by secreting cytokines and growth factors to balance self-renewal and differentiation. Early studies have identified various cytokines for their ability to expand and maintain HSCs *ex vivo*, including SCF, TPO, FLT3-L, and IL-3 et al. [7–9,33,34]. In recent years, many efforts have been placed on screening small molecules to expand HSCs *ex vivo*. Small molecules such as SR1, UM171, CHIR911, valproic acid and so on were of increasing concern with the effect of promoting HSCs self-renewal, multi-lineage potency and homing [10–14,35]. However, previous studies have shown that the expansion effect of some small molecules merely restricted to a certain cell subpopulation, such as SR1 mostly acting on primitive normal hematopoietic progenitors and leukemia stem cells (LSCs) [36], valproic acid (VPA) do not expand LT-HSCs in culture instead maintain the number [37]. Drug combination is likely to represent a key strategy for the future treatment of various diseases, the aim of that is to achieve complementary advantages and reduce adverse effects. Therefore, we wondered if there was a combination that could achieve substantial expansion of LT-HSCs.

Herein we demonstrated that NAM and UM171 had a synergistic effect on the expansion of LT-HSCs, which drive sustained lifelong hematopoiesis. Characterization of the HSCs showed the percentage and absolute number of HSPCs (CD34+CD38-cells), ST-HSCs (CD34+CD38-CD45RA-CD49f+CD90-cells) and LT-HSCs (CD34+CD38-CD45RA-CD49f+CD90+cells) were significantly increased after treatment of NAM+UM171. Specifically, the expansion fold of LT-HSCs in NAM+UM171 treatment group can reach 753.16 ± 83.02, far higher than adding NAM or UM171 alone (100.12 ± 8.82 and 183.64 ± 67.59, respectively). The precise balance of different cell fates (quiescence, self-renewal, differentiation, and apoptosis) acts as the determinant for HSCs numbers. NAM combined with UM171 was more conducive to maintaining the morphology of primitive HSCs, inducing HSCs to enter the cell cycle and reducing the proportion of apoptotic cells. The results indicated that the combination could delay the aging of HSCs, promote cell proliferation and survival. The CFC assays showed that NAM+UM171 increased the number of colony-forming units, especially CFU-GEMM, suggesting that the combination promoted the expansion of multi-potent progenitors.

The results of mechanism exploration indicated that NAM+UM171 treatment led to activation of Notch and Wnt pathways, which have long been recognized as critical factors for the self-renewal of stem cells. Incubation of human UCB-derived $CD34^+$ cells with immobilized Notch ligands increased the number of HSCs and enhanced hematopoietic reconstitution, and downregulation of Notch targets has been found to be associated with differentiation of HSCs. Our experimental results suggested that NAM+UM171 had the potential to significantly enhance the expression of downstream genes in the Notch pathway, such as HES1, HES3, and HEY1. Canonical Wnt signal is associated with self-renewal of various stem cells, including HSCs and embryonic stem cells (ESCs). Researchers proved that Wnt is necessary for normal HSCs function by overexpressing the Wnt-negative regulator DKK1 in osteoblast stem cell niche [38]. Meanwhile, overactivation of the Wnt pathway can lead to the downregulation of the HSCs stemness-related gene HOXB4, further impairing the regenerative capacity, while mild activation of the Wnt pathway does not affect the expression of the gene [39]. Our RT-PCR analysis showed that the expression of key factors in Wnt pathway (AXIN2, MMP7) and HOXB4 gene in HSCs was up-regulated after NAM+UM171 treatment, indicating that the Wnt pathway was mildly activated and could promote HSCs expansion under the combined treatment.

Transcription factors determine specific gene expression patterns in cells to maintain stemness and perform unique functions. Several transcription factors have been found to play key roles in the development and maintenance of HSCs. In addition to HOXB4 mentioned above, the expression levels of other stemness-related genes GATA2 and BMI1 also play a important role during HSCs development and function [40–43]. However, a later study shows that BMI1 is involved in abnormal erythrocytic differentiation [44]. The histone deacetylase SIRT1 is a mediator of genomic and epigenetic maintenance, both of which are critical aspects of stem cell homeostasis. The role of SIRT1 in regulating HSCs biology has been somewhat controversial. Some studies suggested that NAM acts as a SIRT1 inhibitor to prevent HSCs differentiation, in other words, SIRT1 inhibits HSCs function [45]. While some studies found that loss of SIRT1 leads to elevated ROS and DNA damage, that is, SIRT1 is essential for HSCs survival [46,47]. HSCs reside in a low-oxygen niche and maintain low ROS levels. The hypoxic niches are essential to protect HSCs from apoptosis and loss of self-renewal potential. However, with the accumulation of ROS levels, the self-renewal capacity and repopulation ability of HSCs decline [48]. Imbalanced metabolism could result in excessive redox responses, and NAM+UM171 may affect the production of highly reactive chemicals, such as ROS, by upregulating the expression of the antioxidant enzyme CAT.

In conclusion, we found that NAM combined with UM171 can massively expand LT-HSCs *ex vivo*, and the expanded cells maintained the capability of self-renewal and multilineage differentiation. Mechanistic studies suggested that the combination may enhance the antioxidant capacity of HSCs. Meanwhile, NAM+UM171 activated both the Notch and Wnt pathways as well as up-regulated stemness-related genes expression in human HSCs. These observations were consistent with the basic principle of HSCs expansion, which is to induce proliferation without compromising stemness.

Author contribution

YC and YR designed and carried out all experiments, collected and analyzed data and wrote the manuscript. FR and YZ supervised all research. HW supervised all research, contributed to design of the experiments, data analysis and manuscript writing and editing.

Data availability

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

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

We thank Dr Yonghong Wang(Maternity Department,The Second Hospital of Shanxi Medical University)for providing samples. The work is funded by the Science and Technology Department of Shanxi Province (Grant no. YDZJSX2021B009); Health Commission of Shanxi Province (Grant no.2021XM07).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.03.011.

References

[1] Sakurai M, Ishitsuka K, Ito R, Wilkinson AC, Kimura T, Mizutani E, et al. Chemically defined cytokine-free expansion of human haematopoietic stem cells. Nature 2023;615(7950):127–33.
[2] Delaney C, Heimfeld S, Brashem-Stein C, Voorhies H, Manger RL, Bernstein ID. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat Med 2010;16(2):232–6.
[3] Farahzadi R, Fathi E, Mesbah-Namin SA, Vietor I. Granulocyte differentiation of rat bone marrow resident C-kit+ hematopoietic stem cells induced by mesenchymal stem cells could be considered as new option in cell-based therapy. Regen Ther 2023;23:94–101.
[4] Che JLC, Bode D, Kucinski I, Cull AH, Bain F, Becker HJ, et al. Identification and characterization of in vitro expanded hematopoietic stem cells. EMBO Rep 2022;23(10):e55502.
[5] Kent DG, Dykstra BJ, Eaves CJ. Isolation and assessment of single long-term reconstituting hematopoietic stem cells from adult mouse bone marrow. Curr Protoc Stem Cell Biol 2016;38:2A.4.1–2A.4.24.
[6] Futrega K, Atkinson K, Lott WB, Doran MR. Spheroid coculture of hematopoietic stem/progenitor cells and monolayer expanded mesenchymal stem/stromal cells in polydimethylsiloxane microwells modestly improves in vitro hematopoietic stem/progenitor cell expansion. Tissue Eng C Methods 2017;23(4):200–18.
[7] Bozhilov YK, Hsu I, Brown EJ, Wilkinson AC. In vitro human haematopoietic stem cell expansion and differentiation. Cells 2023;12(6):896.
[8] Calvanese V, Nguyen AT, Bolan TJ, Vavilina A, Su T, Lee LK, et al. MLLT3 governs human haematopoietic stem-cell self-renewal and engraftment. Nature 2019;576(7786):281–6.
[9] Zarrabi M, Afzal E, Ebrahimi M. Manipulation of hematopoietic stem cell fate by small molecule compounds. Stem Cell Dev 2018;27(17):1175–90.
[10] Ren Y, Cui Y, Tan Y, Xu Z, Wang H. Expansion strategies for umbilical cord blood haematopoietic stem cells in vitro. Vox Sang 2023;118(11):913–20.
[11] Araki D, Fu JF, Huntsman H, Cordes S, Seifuddin F, Alvarado LJ, et al. NOTCH-mediated ex vivo expansion of human hematopoietic stem and progenitor cells by culture under hypoxia. Stem Cell Rep 2021;16(9):2336–50.
[12] Devine SM, Lazarus HM, Emerson SG. Clinical application of hematopoietic progenitor cell expansion: current status and future prospects. Bone Marrow Transplant 2003;31(4):241–52.
[13] Nikas IP, Paschou SA, Ryu HS. The role of nicotinamide in cancer chemoprevention and therapy. Biomolecules 2020;10(3):477.
[14] Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 2011;333(6039):218–21.
[15] Kent D, Dykstra B, Eaves C. Isolation and assessment of long-term reconstituting hematopoietic stem cells from adult mouse bone marrow. Curr Protoc Stem Cell Biol Chapter 2007;2.
[16] Kent DG, Dykstra BJ, Eaves CJ. Isolation and assessment of single long-term reconstituting hematopoietic stem cells from adult mouse bone marrow. Curr Protoc Stem Cell Biol 2016;38. 2A.4.1–2A.4.24.
[17] Simsek T, Kocabas F, Zheng J, Deberardinis RJ, Mahmoud AI, Olson EN, et al. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. Cell Stem Cell 2010;7(3):380–90.
[18] Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell 2011;9(4):298–310.
[19] Cheung AM, Nguyen LV, Carles A, Beer P, Miller PH, Knapp DJ, et al. Analysis of the clonal growth and differentiation dynamics of primitive barcoded human cord blood cells in NSG mice. Blood 2013;122(18):3129–37.
[20] Li Y, He M, Zhang W, Liu W, Xu H, Yang M, et al. Expansion of human megakaryocyte-biased hematopoietic stem cells by biomimetic Microniche. Nat Commun 2023;14(1):2207.
[21] Ren Y, Cui Y, Feng J, Tan Y, Ren F, Zhang Y, et al. Synergistic effect and molecular mechanism of PVA and UM171 in ex vivo expansion of primitive hematopoietic stem cells. J Cell Biochem 2024;125(1):79–88.
[22] Son MJ, Son MY, Seol B, Kim MJ, Yoo CH, Han MK, et al. Icotinamide overcomes pluripotency deficits and reprogramming barriers. Stem cells 2013;31(6):1121–35.
[23] Horwitz ME, Chao NJ, Rizzieri DA, Long GD, Sullivan KM, Gasparetto C, et al. Umbilical cord blood expansion with nicotinamide provides long-term multilineage engraftment. J Clin Invest 2014;124(7):3121–8.
[24] Al-Amoodi AS, Li Y, Al-Ghuneim A, Allehaibi H, Isaioglou I, Esau LE, et al. Refining the migration and engraftment of short-term and long-term HSCs by enhancing homing-specific adhesion mechanisms. Blood Adv 2022;6(15):4373–91.
[25] Li X, Zeng X, Xu Y, Wang B, Zhao Y, Lai X, et al. Mechanisms and rejuvenation strategies for aged hematopoietic stem cells. J Hematol Oncol 2020;13(1):31.
[26] de Haan G, Lazare SS. Aging of hematopoietic stem cells. Blood 2018;131(5):479–87.
[27] Kowalczyk MS, Tirosh I, Heckl D, Rao TN, Dixit A, Haas BJ, et al. Single-cell RNA-seq reveals changes in cell cycle and differentiation programs upon aging of hematopoietic stem cells. Genome Res 2015;25(12):1860–72.
[28] Boyer SW, Rajendiran S, Beaudin AE, Smith-Berdan S, Muthuswamy PK, Perez-Cunningham J, et al. Clonal and quantitative in vivo assessment of hematopoietic stem cell differentiation reveals strong erythroid potential of multipotent cells. Stem Cell Rep 2019;12(4):801–15.
[29] Park JA, Park S, Park WY, Han MK, Splitomicin Lee Y. A SIRT1 inhibitor, enhances hematopoietic differentiation of mouse embryonic stem cells. Int J Stem Cells 2019;12(1):21–30.
[30] Li C, Wu B, Li Y, Liu Y, Wang J, Xie J, et al. Loss of sphingosine kinase 2 promotes the expansion of hematopoietic stem cells by improving their metabolic fitness. Blood 2022;140(15):1686–701.
[31] Mochizuki-Kashio M, Shiozaki H, Suda T, Nakamura-Ishizu A. Mitochondria turnover and lysosomal function in hematopoietic stem cell metabolism. Int J Mol Sci 2021;22(9):4627.
[32] Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell 2000;100(1):157–68.
[33] Miller CL, Audet J, Eaves CJ. Ex vivo expansion of human and murine hematopoietic stem cells. Methods Mol Med 2002;63:189–208.
[34] Zhang CC, Lodish HF. Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. Blood 2005;105(11):4314–20.
[35] Zimran E, Papa L, Hoffman R. Ex vivo expansion of hematopoietic stem cells: finally transitioning from the lab to the clinic. Blood Rev 2021;50:100853.
[36] Fares I, Rivest-Khan L, Cohen S, Sauvageau G. Small molecule regulation of normal and leukemic stem cells. Curr Opin Hematol 2015;22(4):309–16.
[37] Mahmud N, Petro B, Baluchamy S, Li X, Taioli S, Lavelle D, et al. Differential effects of epigenetic modifiers on the expansion and maintenance of human cord blood stem/progenitor cells. Biol Blood Marrow Transplant 2014;20(4):480–9.
[38] Fleming HE, Janzen V, Lo Celso C, Guo J, Leahy KM, Kronenberg HM, et al. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. Cell Stem Cell 2008;2(3):274–83.
[39] Luis TC, Naber BA, Roozen PP, Brugman MH, de Haas EF, Ghazvini M, et al. Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. Cell Stem Cell 2011;9(4):345–56.
[40] de Pater E, Kaimakis P, Vink CS, Yokomizo T, Yamada-Inagawa T, van der Linden R, et al. Gata2 is required for HSC generation and survival. J Exp Med 2013;210(13):2843–50.
[41] Peters IJA, de Pater E, Zhang W. The role of GATA2 in adult hematopoiesis and cell fate determination. Front Cell Dev Biol 2023;11:1250827.
[42] Rizo A, Dontje B, Vellenga E, de Haan G, Schuringa JJ. Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. Blood 2008;111(5):2621–30.
[43] Lv K, Tong W. A protein synthesis brake for hematopoietic stem cell maintenance. Genes Dev 2022;36(15–16):871–3.
[44] Zhou T, Wang L, Zhu KY, Dong M, Xu PF, Chen Y, et al. Dominant-negative C/ebpα and polycomb group protein Bmi1 extend short-lived hematopoietic stem/progenitor cell life span and induce lethal dyserythropoiesis. Blood 2011;118(14):3842–52.
[45] Peled T, Shoham H, Aschengrau D, Yackoubov D, Frei G, Rosenheimer GN, et al. Nicotinamide, a SIRT1 inhibitor, inhibits differentiation and facilitates expansion of hematopoietic progenitor cells with enhanced bone marrow homing and engraftment. Exp Hematol 2012;40(4):342–55. e1.
[46] Bigarella CL, Liang R, Ghaffari S. Stem cells and the impact of ROS signaling. Development 2014;141(22):4206–18.
[47] Ghaffari S. Haematopoietic stem cell quiescence exposed using mitochondrial membrane potential. Curr Opin Hematol 2022;30(1):1–3.
[48] Li X, Zeng X, Xu Y, Wang B, Zhao Y, Lai X, et al. Mechanisms and rejuvenation strategies for aged hematopoietic stem cells. J Hematol Oncol 2020;13(1):31.