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***Basic Study***

**Effects of different concentrations of nicotinamide on hematopoietic stem cells cultured *in vitro***

Ren Y *et al*. Concentration of NAM affects HSC expansion

Yan Ren, Yan-Ni Cui, Hong-Wei Wang

**Yan Ren, Yan-Ni Cui,** The Second Clinical Medical College, Shanxi Medical University, Taiyuan 030001, Shanxi Province, China

**Yan Ren, Yan-Ni Cui, Hong-Wei Wang,** Department of Hematology, The Second Hospital of Shanxi Medical University, Taiyuan 030001, Shanxi Province, China

**Hong-Wei Wang,** Department of Hematology, Key Laboratory of Molecular Diagnosis and Treatment of Blood Diseases in Shanxi Province, Taiyuan 030001, Shanxi Province, China

**Co-first authors:** Yan Ren and Yan-Ni Cui.

**Author contributions:** Ren Y and Cui YN designed and coordinated the study; Ren Y performed experiments and wrote the manuscript; Cui YN acquired and analyzed the data; Wang HW contributed to ideas, supervision, review and editing; and all authors approved the final version of the article.

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**Corresponding author: Hong-Wei Wang, PhD, Professor,** Department of Hematology, The Second Hospital of Shanxi Medical University, No. 382 Wuyi Road, Taiyuan 030001, Shanxi Province, China. wanghw68@hotmail.com

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**Abstract**

BACKGROUND

*In vitro* expansion to increase numbers of hematopoietic stem cells (HSCs) in cord blood could improve clinical efficacy of this vital resource. Nicotinamide (NAM) can promote HSC expansion *ex vivo*, but its effect on hematopoietic stem and progenitor cells (HSPCs, CD34+CD38−) and functional subtypes of HSCs – short-term repopulating HSCs (ST-HSCs, CD34+CD38−CD45RA−CD49f+) and long-term repopulating HSCs (LT-HSCs, CD34+CD38−CD45RA−CD49f+CD90+) is not yet known. As a sirtuin 1 (SIRT1) inhibitor, NAM participates in regulating cell adhesion, polarity, migration, proliferation, and differentiation. However, SIRT1 exhibits dual effects by promoting or inhibiting differentiation in different tissues or cells. We propose that the concentration of NAM may influence proliferation, differentiation, and SIRT1 signaling of HSCs.

AIM

To evaluate the effects and underlying mechanisms of action of different concentrations of NAM on HSC proliferation and differentiation.

METHODS

CD34+ cells were purified from umbilical cord blood using MacsCD34 beads, and cultured for 10–12 d in a serum-free medium supplemented with cytokines, with different concentrations of NAM added according to experimental requirements. Flow cytometry was used to detect phenotype, cell cycle distribution, and apoptosis of the cultured cells. Real-time polymerase chain reaction was used to detect the transcription levels of target genes encoding stemness-related factors, chemokines, components of hypoxia pathways, and antioxidant enzymes. Dichloro-dihydro-fluorescein diacetate 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

Compared with the control group, the proportion and expansion folds of HSPCs (CD34+CD38−) incubated with 5 mmol/L or 10 mmol/L NAM were significantly increased (all *P* < 0.05). The ST-HSCs ratio and fold expansion of the 5 mmol/L NAM group were significantly higher than those of the control and 10 mmol/L NAM groups (all *P* < 0.001), whereas the LT-HSCs ratio and fold expansion of the 10 mmol/L NAM group were significantly higher than those of the other two groups (all *P* < 0.05). When the NAM concentration was > 10 mmol/L, cell viability significantly decreased. In addition, compared with the 5 mmol/L NAM group, the proportion of apoptotic cells in the 10 mmol/L NAM group increased and the proportion of cells in S and G2 phase decreased. Compared with the 5 mmol/L NAM group, the HSCs incubated with 10 mmol/L NAM exhibited significantly inhibited SIRT1 expression, increased intracellular ROS content, and downregulated expression of genes encoding antioxidant enzymes (superoxide dismutase 1, peroxiredoxin 1).

CONCLUSION

Low concentrations (5 mmol/L) of NAM can better regulate the balance between proliferation and differentiation, thereby promoting expansion of HSCs. These findings allow adjustment of NAM concentrations according to expansion needs.

**Key Words:** Hematopoietic stem cells; Nicotinamide; Concentration; Proliferation; Differentiation; Sirtuin 1

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**Core tip:** This study reveals the dominant subgroups of hematopoietic stem cells (HSCs) and molecular mechanisms underlying the effects of different nicotinamide (NAM) concentrations. Activation or inhibition of sirtuin 1 (SIRT1) is determined by the concentration of NAM. High concentrations inhibit SIRT1 but are not conducive to self-renewal of HSCs, whereas low concentrations balance HSC proliferation and differentiation by regulating the SIRT1–HIF1A pathway and reactive oxygen species production, effectively promoting *in vitro* expansion of the stem cells. These findings could allow adjustment of NAM concentrations according to expansion needs and may help predict small molecules that synergistically promote expansion with NAM.

**INTRODUCTION**

Hematopoietic stem cells (HSCs) can self-renew and give rise to mature cells of all hematopoietic lineages following extensive proliferation and differentiation[1,2]. Umbilical cord blood (UCB) is an important source of HSCs because it can be obtained through noninvasive means, has low requirements for the matching of human leukocyte antigens, and has a low incidence of chronic graft-versus-host disease. However, the limited cell dose in a single UCB sample can lead to delayed engraftment after transplantation[3]. Obtaining a sufficient number of HSCs through *ex vivo* expansion has the potential to overcome such challenges.

Small molecules represent novel modalities for *ex vivo* expansion of HSCs[4-6], and the roles of different molecules in HSC transplantation are diverse. As a form of vitamin B-3, nicotinamide (NAM) can delay differentiation and maintain stemness, as well as promote neutrophil recovery in patients after transplantation[7-9]. In humans, CD34+CD38− cells are classic hematopoietic stem and progenitor cells (HSPCs), and HSCs can be functionally divided into short-term repopulating HSCs (ST-HSCs, CD34+CD38−CD45RA−CD49f+) and long-term repopulating HSCs (LT-HSCs, CD34+CD38−CD45RA−CD49f+CD90+)[10-12]. Preclinical models indicate that the recovery of blood cells immediately after transplantation is repopulated primarily by ST-HSCs while long-term sustenance of trilineage hematopoiesis is the contribution of LT-HSCs that have the ability to self-renew. LT-HSCs drive sustained lifelong hematopoiesis, these cells are relatively quiescent, hence resulting in a prolonged period of leukopenia[10-15]. Thus, the primary goal of UCB expansion is twofold; namely, to reduce the time taken to achieve blood cell reconstitution following transplantation, and ensuring sustained long-term trilineage hematopoiesis. NAM promotes *in vitro* expansion of HSPCs, but its effects on ST-HSCs and LT-HSCs have yet to be elucidated. Emerging studies suggest that NAM mediates the metabolic transition of aging stem cells by upregulating the expression of young genes such as sirtuin 1 (SIRT1), thereby maintaining stemness[16-22]. This contradicts the viewpoint that NAM acts as a SIRT1 inhibitor[23]. Furthermore, SIRT1 exhibits a dual effect of promoting or inhibiting differentiation in different tissues or cells[24]. Based on these observations, we propose that the proliferation, differentiation, and SIRT1 signaling pathway of HSCs may be influenced by the concentration of NAM.

In this study, we evaluated the effects of different concentrations of NAM on the proliferation and differentiation of HSCs during *in vitro* culture. Proliferation is reflected by fold expansion of total nucleated cells (TNCs) and differentiation is reflected by phenotype analysis of HSPC, ST-HSC and LT-HSC populations. Apoptosis ratio and cell cycle distribution were used to reveal the influencing factors of cell quantity differences; reactive oxygen species (ROS) production and cytokine levels were evaluated to reveal the relevant factors of differentiation differences; and real-time polymerase chain reaction (RT-PCR) detection of genes related to stemness, antioxidant enzymes, and homing were used to explore the molecular mechanisms underlying the actions of NAM. NAM at 5 mmol/L and 10 mmol/L could inhibit differentiation and maintain HSPCs, with the higher concentration being more conducive to the maintenance of LT-HSCs. However, owing to significant proapoptotic effects, this high concentration of 10 mmol/L NAM reduced cell survival and self-renewal, whereas the lower concentration of 5 mmol/L of NAM could better regulate the balance between proliferation and differentiation, thereby promoting the effective expansion of HSCs. In general, the differential sensitivities of LT-HSCs and ST-HSCs to NAM may be used to improve the outcome of *ex vivo* expansion.

**MATERIALS AND METHODS**

***Sample preparation and isolation of CD34+ cells from human UCB***

A gynecologist collected UCB samples from consenting donors according to ethical procedures approved by the Second Hospital of Shanxi Medical University (Shanxi, China). UCB mononuclear cells (MNCs) were isolated by Ficoll (Tianjin Haoyang Biological Products Technology) and density-gradient centrifugation. Specifically, UCB depleted of erythrocytes (by adding hydroxyethyl starch) was slowly pipetted on Ficoll at a ratio of 2:1 and centrifuged (2000 rpm, 20 min), followed by collection of the interface layer as MNCs. The MNCs were washed and suspended in column buffer [phosphate buffered saline (PBS) and 0.5% bovine serum albumin; Gibco], then CD34+ cells labeled with CD34 MicroBeads (Miltenyi Biotec) were separated out by loading the cell suspension onto LS Columns (Miltenyi Biotec) associated with a magnetic field.

***Cell culture***

Before being seeded onto 24-well plates (Corning), CD34+ cells were resuspended in serum-free medium (5 × 104/mL), which was composed of Iscove Modified Dulbecco Medium (IMDM; Gibco), supplemented with 10 ng/mL human stem cell factor (Miltenyi Biotec), 100 ng/mL thrombopoietin (Miltenyi Biotec), 1% penicillin–streptomycin–glutamine (Gibco), and NAM at different concentrations. According to relevant research data[25,26] and preliminary experimental results (Supplementary Figure 1), we set the concentration of NAM to 5 mmol/L, 10 mmol/L,or 15 mmol/L. Based on the different concentrations of NAM, the cells were divided into the control group (no NAM), low concentration group (5 mmol/L NAM), and high concentration group (10 mmol/L NAM). Cells were incubated in a humidified incubator at 37 °C, 5% CO2, and 5% O2 for 10–12 d.

***Cell survival rate and cell size detection***

Trypan Blue cell viability counting was performed. Trypan Blue (Thermo Fisher Scientific) can only pass through an incomplete cell membrane, and dead cells were dyed clear blue. After mixing 10 μL cell suspension with 10 μL Trypan Blue, the viability and size of the cells in suspension were measured by a Countess 3 Automated Cell Counter (Thermo Fisher Scientific).

***Phenotypic analysis***

After incubation with NAM for 10–12 d, the cells were collected and washed with PBS. Next, the cells were stained in PBS supplemented with the following antibody and fluorophore combinations for 30 min at 4°C: ECD-labeled anti-human CD34 (IM2709U; Beckman), FITC-labeled anti-human CD38 (A07778; Beckman), Pacific Blue-labeled anti-human CD45RA (A82946; Beckman), APC-CY7-labeled anti-human CD49f (313628; Biolegend), and PerCP-CY5-labeled anti-human CD90 (IM3703; Beckman). After a washing step, the stained cells were analyzed by Navios flow cytometer (Beckman Coulter). For each sample, at least 30000 events were recorded. Isotype controls were also prepared for every experiment.

***Colony-forming cell assay***

According to the cell count, we adjusted the concentration of CD34+ cells of each group to 105/mL, add 50 μL cells to 450 μL IMDM (Gibco) for mixing, and added 150 μL cells to 1.5 mL MethodCultTM medium (Stem Cell Technologies). We connected a 1.6-mm needle to a 2-mL disposable sterile syringe and distributed the MethodCultTM mixture containing cells into a 35-mm culture dish (Corning), so that the medium was evenly distributed on the surface of each dish. The cells were cultured in 5% CO2 at 37 °C for 14 d, and culture dishes were visually scored for colony-forming unit (CFU)-granulocyte/macrophage, CFU-erythrocyte (CFU-E) and CFU-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM).

***Apoptosis detection***

The cultured cells were collected and washed twice with precooled PBS. Cells were stained for 15 min at room temperature with PE-Annexin V Apoptosis Detection Kit (BD Biosciences). Specifically, 5 μL PE-Annexin V and 5 μL 7-amino-actinomycin D were added to each sample. Samples were analyzed by Navios flow cytometer within 1 h.

***Cell cycle analysis***

The cultured cells were washed with precooled PBS, resuspended in 70% ethanol at 4°C for 2–6 h, and washed with PBS and incubated with 0.4 mL propidium iodide staining solution (Biosciences) for 30 min at 37°C in the dark. The 0.4 mL of propidium iodide staining solution was composed of 384 μL staining buffer, 15 μL 25× propidium solution, and 1 μL RNase (10 mg/mL). A minimum of 40 000 cells were collected at a low speed for each sample.

***ROS detection***

The cultured cells were resuspended at a density of 5 × 105 to 1 × 106 cells/mL, and the dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe was diluted with serum-free medium (Beyotime Biotechnology). The cells were resuspended in diluted DCFH-DA and incubated for 20 min in a 37°C incubator. The cells were mixed upside down every 3–5 min to ensure full contact between the probe and the cell. The cells were washed with serum-free medium three times to fully remove DCFH-DA that had not entered the cells. The fluorescence intensity of ROS was measured within 2 h by Navios flow cytometer. The median level of mean fluorescence intensity of ROS was obtained in Kaluza software (Beckman) for each sample after adjusting them to the same measuring cell number.

***RT-PCR***

Total RNA was extracted from the cells cultured with or without NAM by the Trizol method. For cDNA synthesis, total RNA was reverse transcribed with a cDNA synthesis kit (Bio-Rad). PCR was performed using a SYBR Premix Ex TaqTMII (Tli RNaseH Plus; TaKaRa) and the CFX96 RT-PCR detection system (Bio-Rad). Each reaction was repeated at least three times to demonstrate reproducibility, and data were analyzed using the CFX96 Real-Time System. Normalized values were obtained by subtracting the threshold cycle (Ct) of GAPDH 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: CD34 forward (5’-CTACAACACCTAGTACCCTTGGA-3’) and reverse (5’-GGTGAACACTGTGCTGATTACA-3’); CD133 forward (5’-AGTCGGAAACTGGCAGATAGC-3’) and reverse (5’-GGTAGTGTTGTACTGGGCCAAT-3’); CXCR4 forward (5’-GGGCAATGGATTGGTCATCCT-3’) and reverse (5’-TGCAGCCTGTACTTGTCCG-3’); SIRT1 forward (5’-CAAACTTTGCTGTAACCCTGT-3’) and reverse (5’-CAGCCACTGAAGTTCTTTCAT-3’); HIF1A forward (5’-GGTCTAGGAAACTCAAAACCTGA-3’) and reverse (5’-TCCTCACACGCAAATAGCTGA-3’); superoxide dismutase (SOD)1 forward (5’-GGTGGGCCAAAGGATGAAGAG-3’) and reverse (5’-CCACAAGCCAAACGACTTCC-3’); SOD2 forward (5’-GCTCCGGTTTTGGGGTATCTG-3’) and reverse (5’-GCGTTGATGTGAGGTTCCAG-3’); peroxiredoxin 1 (PRDX1) forward (5’-CCACGGAGATCATTGCTTTCA-3’) and reverse (5’-AGGTGTATTGACCCATGCTAGAT-3’); β-actin forward (5’-AGAGCTACGAGCTGCCTGAC-3’) and reverse (5’-AGCACTGTGTTGGCGTACAG-3’).

***Cytokine assay***

This assay was conducted according to the Instruction Manual of BD™ Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (Catalog No. 560484). The fresh cytokine standards were prepared to run with each experiment. We added 50 μL sample and 50 μL 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. Data were analyzed using FCAP Array software.

***Statistical analysis***

The significant differences between each group were analyzed using SPSS 22.0 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. The values were plotted as the mean ± SD. Probability values *P* < 0.05 were considered significant.

**RESULTS**

***Effect of different concentrations of NAM on the quality of HSCs***

We measured the proportion of CD34+CD38− (HSPCs), CD34+CD38−CD45RA−CD49f+ (ST-HSCs), and CD34+CD38−CD45RA−CD49f+CD90+ (LT-HSCs) subsets in the control group (no NAM) and different NAM groups (5, 10 and 15 mmol/L). The proportion of HSPCs in the control group was 14.28% ± 0.36%, while the 5 mmol/L and 10 mmol/L NAM groups accounted for 15.03% ± 0.36% and 15.37% ± 0.46% respectively, which was significantly higher than in the control group (all *P* < 0.05), whereas there was no significant difference between the first two groups (Figure 1A). The proportion of ST-HSCs was highest in the 5 mmol/L NAM group (9.51% ± 0.35%), followed by the 10 mmol/L NAM group (4.33% ± 0.31%) (*P* < 0.001), and both groups had significantly higher proportions of ST-HSCs than the control group (3.63% ± 0.11%) (all *P* < 0.05) (Figure 1B). The proportion of LT-HSCs in the 10 mmol/L NAM group (1.26% ± 0.16%) was significantly higher than that in the control group (0.39% ± 0.04%) and the 5 mmol/L NAM group (0.34% ± 0.03%), and there was no significant difference between the latter two groups (Figure 1C). The lower the differentiation and senescence of stem cells, the smaller their diameter[27], and the results of cell size testing in each group confirmed (Figure 1D). Cell viability for the 15 mmol/L NAM group was poor and the cultures contained a lot of cell debris, which led to nonspecific antibody binding that was unsuitable for phenotype analysis (Figure 1E). In addition, the results of the CFU assay show that the total number of colonies and the number of CFU-E and CFU-GEMM in the 5 mmol/L NAM group was significantly higher than in the control group, while the total colony-forming ability of the 10 mmol/L NAM group was impaired compared to the control group, but the number of CFU-GEMM still significantly increased (Figure 1F and G). These results are consistent with other reports that NAM treatment can inhibit the differentiation of HSCs[8].

***Effect of different concentrations of NAM on the quantity of HSCs***

Fold expansion of TNCs and the degree of cell differentiation were reliable indicators for evaluating the *ex vivo* expansion efficiency of HSCs. Under the microscope, small and round HSCs were visible, and the number of cells in the 15 mmol/L NAM group was significantly lower than in the other groups (Figure 2A). The 5 mmol/L NAM group had the highest fold expansion of TNCs, followed by the control group; both of which were significantly higher than the 10 mmol/L and 15 mmol/L NAM groups, with the 15 mmol/L NAM group having the lowest fold expansion of TNCs (Figure 2B). The fold expansion of HSPCs in the 5 mmol/L (107.25 ± 2.71) and 10 mmol/L (100.65 ± 2.13) NAM groups was significantly higher than that in the control group (61.10 ± 2.12) (all *P* < 0.001) (Figure 2C). The fold expansion of ST-HSCs was highest in the 5 mmol/L NAM group (122.17 ± 4.09) and was significantly higher than in the control (48.11 ± 1.54) and 10 mmol/L NAM (30.82 ± 2.50) groups (all *P* < 0.001) (Figure 2D). Fold expansion of LT-HSCs was highest in the 10 mmol/L NAM group, and was significantly higher than in the control and 5 mmol/L NAM groups (all *P* < 0.005) (Figure 2E).

***Effect of different concentrations of NAM on HSC apoptosis and cell cycle distribution***

To investigate whether NAM affected other biological behaviors of HSCs, we measured the proportion of apoptotic cells and cell cycle distribution in each group. The proportion of apoptotic cells in the 10 mmol/L NAM group was significantly higher than in the control and 5 mmol/L NAM groups, with a significant decrease in the proportion in the control group compared with the 5 mmol/L NAM group (all *P* < 0.001) (Figures 3A and B). In addition, the proportion of cells in S and G2 phase in the 5 mmol/L NAM group was significantly higher than those in the control and 10 mmol/L NAM groups, with a significant decrease in the proportion in the control group compared with the 10 mmol/L NAM group (Figure 3C). This indicated that the effect of NAM on HSC proliferation and apoptosis was concentration dependent. The high proportion of apoptosis in the 10 mmol/L NAM group may be one of the reasons for its impaired overall colony-formation ability.

***Molecular mechanism of NAM affecting the proliferation and differentiation of HSCs***

*In vivo*, primitive HSCs can be distinguished by their low mitochondrial activity, as they primarily rely on glycolytic metabolism as their energy source to maintain a dormant state. *Ex vivo* culture conditions cannot completely mimic the niche of HSCs, but do drive metabolic transitions toward oxidative phosphorylation and activation of inflammatory pathways, leading to an increase in ROS and inflammatory cytokines, which results in rapid differentiation[28-33]. In this study, compared with freshly isolated CD34+ cells, the control and NAM groups exhibited a significant increase in intracellular ROS levels, with the control group having the highest ROS level and the NAM groups having a significantly lower ROS content than the control group (Figure 4A). Additionally, the concentration of interleukin-6 in the cell supernatant was significantly increased, with that of the 5 mmol/L and 10 mmol/L NAM groups not exceeding 3 ng/mL and being significantly lower than that of the control group (Figure 4B).

The expression of stemness, chemotaxis, hypoxia pathway and antioxidant enzyme genes are common indicators for exploring the mechanisms of action of different small molecule compounds in HSC amplification systems[34-39]. To further explore the molecular mechanisms by which NAM affects the proliferation and differentiation of HSCs, we compared the expression of genes encoding stemness-related factors, chemokines, components of hypoxia pathways, and antioxidant enzymes in each group of cells (Figure 4C). The hypoxia pathway SIRT1–HIF1A was significantly upregulated in the 5 mmol/L NAM group compared with the other two groups. Compared with the control group, the 5 mmol/L NAM group showed significant upregulation of antioxidant enzymes PRDX1 and SOD1, consistent with the results of the ROS detection mentioned above. In addition, compared with the control and 5 mmol/L NAM groups, the 10 mmol/L NAM group had significantly inhibited SIRT1 expression. The expression of the chemokine CXCR4 was significantly increased in the 5 mmol/L and 10 mmol/L NAM groups compared with the control group.

**DISCUSSION**

The basic HSCs *in vitro* culture consists of a serum-free medium supplemented with cytokines, and the addition of small molecule substances can significantly enhance amplification efficiency[15,40]. For example, NAM can significantly increase the proportion of CD34+CD38− cells (HSPCs). However, the CD34+CD38− phenotype is an unpredictable surface marker for *in vivo* repopulation of cell cultures, resulting in separation of phenotype from *in vivo* hematopoietic reconstitution ability[41,42]. According to reconstruction dynamics in primary and secondary recipients after transplantation, HSCs can be divided into LT-HSCs and ST-HSCs. LT-HSCs are believed to be located at the top of the hematopoietic system and can differentiate downstream to produce ST-HSCs, pluripotent progenitor cells, lineage-specific progenitor cells, and terminally differentiated hematopoietic cells. LT-HSCs can provide delayed but persistent hematopoiesis, whereas ST-HSCs contribute to early and temporary hematopoiesis recovery[43,44]. Therefore, compared with HSPCs, these two subpopulations can more accurately evaluate the *in vivo* hematopoietic reconstitution ability and the expansion efficiency of NAM on HSCs.

In this study, we evaluated the amplification efficiency of different concentrations of NAM on HSPCs, ST-HSCs, and LT-HSCs, and found that 5 mmol/L and 10 mmol/L NAM are beneficial for maintaining HSPCs. The lower concentration (5 mmol/L) of NAM was more conducive to expansion of ST-HSCs, whereas the higher concentration (10 mmol/L) had a more significant effect on promoting expansion of LT-HSCs. Based on these findings, the working concentration of NAM can be adjusted according to the expansion requirements.

The key to promoting self-renewal of HSCs lies in regulating the balance between proliferation and differentiation, which requires activating dormant stem cells to enter the proliferative cell cycle while also preventing excessive oxidative phosphorylation and ROS production. For example, the HSC self-renewal agonist UM171 stimulates *ex vivo* HSC expansion by establishing a critical balance between proinflammatory and anti-inflammatory mediators[45].

In our research, low concentrations of NAM caused HSCs to exit G0 phase and enter the proliferative cell cycle, leading to an increase in cell numbers. In addition, the low concentrations of NAM inhibited differentiation by reducing ROS production. This indicates that low concentrations of NAM can better regulate the balance between proliferation and differentiation, thereby promoting effective expansion of HSCs. When the concentration of NAM exceeds 10 mmol/L, apoptosis and cell necrosis increase, which is not conducive to the survival and self-renewal of HSCs. The high intracellular ROS levels detected in our 10 mmol/L NAM group contradict the fact that high concentrations of NAM are beneficial for maintaining LT-HSCs. This may be due the cytotoxicity of high concentrations of NAM, and this was confirmed by the significant increase in apoptotic cells and significant decrease in cell viability in the 10 mmol/L and 15 mmol/L NAM groups.

Activating SIRT1 can protect cells from oxidative stress damage and regulate cell proliferation by upregulating the SIRT1–HIF1A pathway[46,47], whereas inhibiting SIRT1 may be related to delaying differentiation[48]. NAM is considered a SIRT1 inhibitor that delays stem cell differentiation. We measured the transcription levels of SIRT1 in each group of cells and found that compared with the control group, SIRT1 was significantly upregulated in the 5 mmol/L NAM group and significantly downregulated in the 10 mmol/L NAM group. This indicates that NAM can only inhibit SIRT1 expression at high concentrations, whereas low concentrations of NAM do not inhibit SIRT1 but may upregulate transcription of this crucial regulator. Activation of SIRT1 may promote cell proliferation by upregulating the SIRT1–HIF1A pathway, which is consistent with the higher proportion of S phase cells and fold expansion of TNCs observed in the 5 mmol/L NAM group in our above results.

**CONCLUSION**

Different concentrations of NAM have distinct effects on proliferation and differentiation of HSCs. Although a high concentration of NAM is more conducive to the expansion of LT-HSCs, the cells are affected by oxidative stress and apoptosis, which may negatively impact hematopoietic reconstitution after transplantation. Low concentrations of NAM can better regulate the balance between proliferation and differentiation, thereby promoting effective expansion of HSCs. In addition, the transcription level of SIRT1 is correlated with the concentration of NAM. Upregulation of SIRT1–HIF1A can promote proliferation, whereas inhibition of SIRT1 may achieve a delayed differentiation effect.

**ARTICLE HIGHLIGHTS**

***Research background***

The *in vitro* expansion strategy of increasing the number of hematopoietic stem cells (HSCs) in cord blood is expected to improve its clinical efficacy. Nicotinamide (NAM) is one of the small molecules that can promote the expansion of hematopoietic stem and progenitor cells (HSPCs) *ex vivo*, but its mechanism of action and its effects on short-term repopulating HSCs (ST-HSCs) and long-term repopulating HSCs (LT-HSCs) have not yet been established. Revealing the dominant subgroups of NAM and elucidating the relevant mechanisms not only helps to recommend the working concentration of NAM according to demand, but also helps to reveal the signaling pathways involved in the regulation of HSC proliferation and differentiation.

***Research motivation***

The effects of different concentrations of NAM on proliferation and differentiation of HSCs, as well as whether it affects sirtuin 1 (SIRT1) transcription levels, have not been reported. There are various small molecule substances used for *in vitro* culture of HSCs (including UM171, SR1, VPA, NAM and ID8), which may affect the maintenance, proliferation, differentiation and homing of HSCs by regulating different pathways, and different molecular pathways may have synergistic effects. This study aimed to provide a theoretical basis for the future joint application of multiple small molecules by elucidating the molecular mechanism of NAM. Our work is crucial for selecting the working concentration of NAM and predicting small molecules that have a synergistic effect with NAM.

***Research objectives***

To evaluate the effects and mechanisms of different concentrations of NAM on HSC proliferation and differentiation.

***Research methods***

In this study, we added different concentrations of NAM to serum-free culture medium inoculated with CD34+ cells. We then measure the number, molecular phenotype, cycle distribution, and apoptosis of each group of cells and explore the mechanism by detecting the levels of reactive oxygen species (ROS), inflammatory factors and related gene transcription.

***Research results***

We evaluated the expansion efficiency of different concentrations of NAM on HSPCs, ST-HSCs as well as LT-HSCs, and the results showed that 5 mmol/L and 10 mmol/L NAM were beneficial for maintaining HSPCs, with low concentrations (5 mmol/L) of NAM being more conducive to ST-HSCs expansion, while high concentrations (10 mmol/L) of NAM had a more significant effect on promoting LT-HSCs expansion. Low concentrations of NAM can better regulate the balance between proliferation and differentiation, thereby promoting effective expansion of HSCs.

***Research conclusions***

Low concentration of NAM did not inhibit but upregulated the transcription of SIRT1, promoting cell proliferation by activating the SIRT1–HIF1A pathway, delaying stem cell differentiation by increasing ROS clearance, and ultimately promoting effective expansion of HSCs.

***Research perspectives***

Drugs that specifically target LT-HSCs or ST-HSCs may help in the development of tailored HSC grafts that either facilitate sustained blood cell reconstitution or rapidly improve blood cell counts as a bridge after anticancer radiotherapy or chemotherapy. Our findings could allow adjustment of NAM concentrations according to expansion needs and may help predict small molecules that synergistically promote expansion with NAM.

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**Footnotes**

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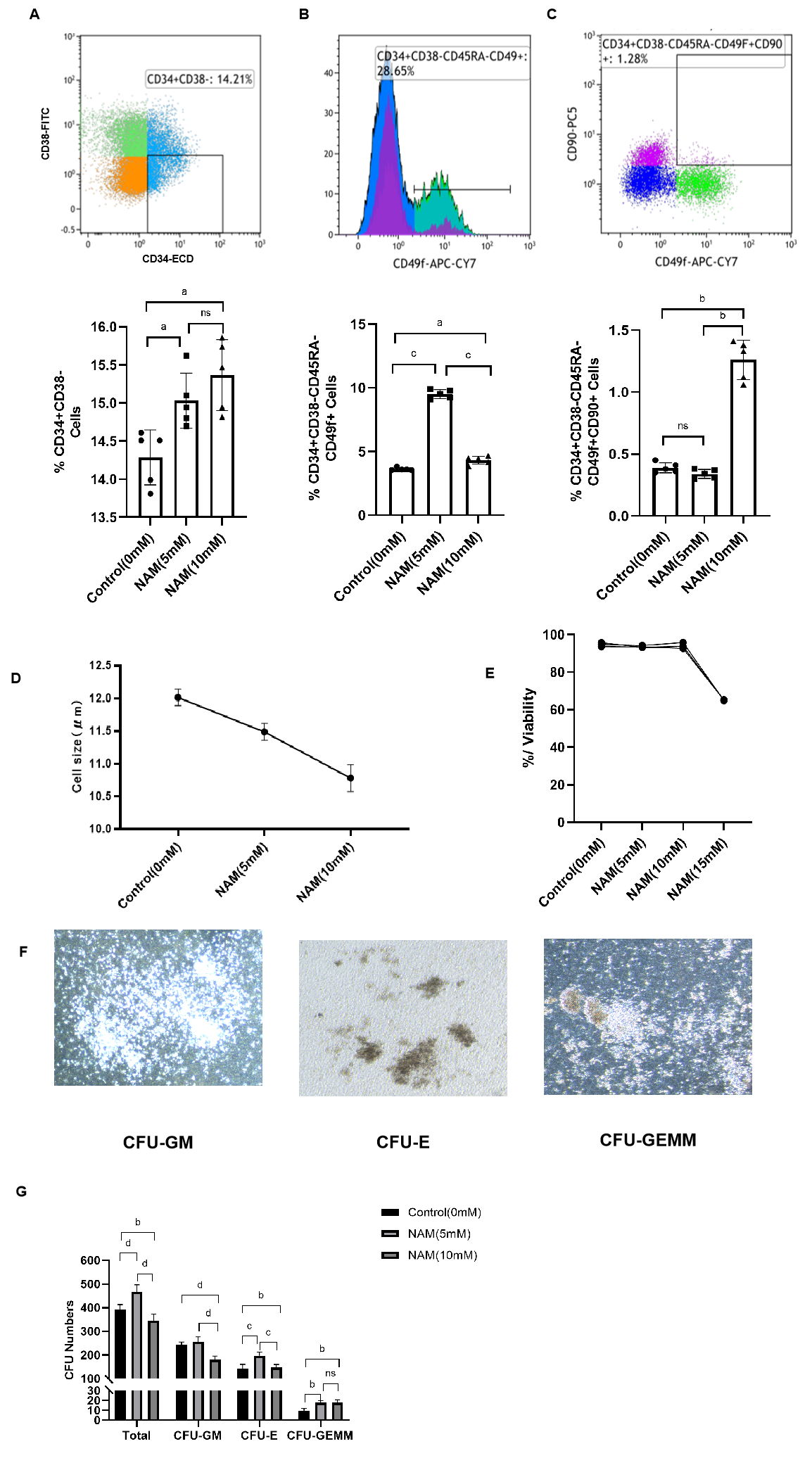
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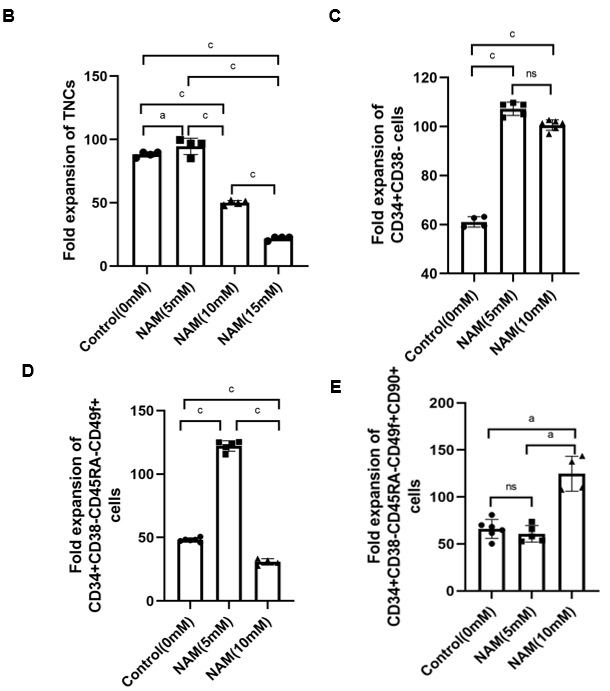
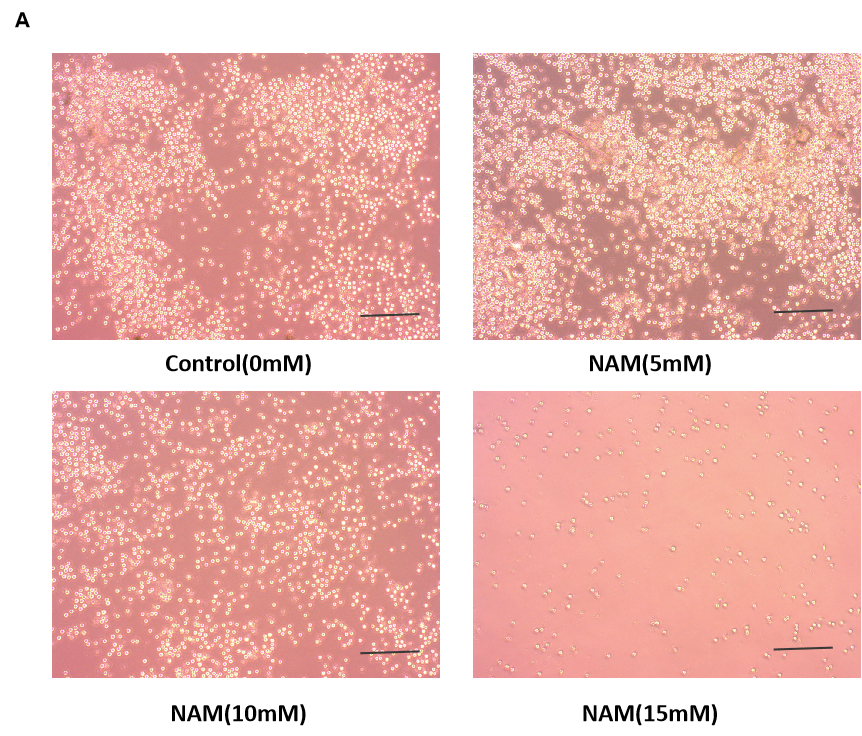
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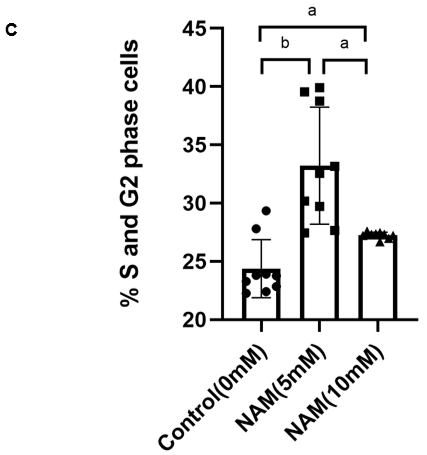
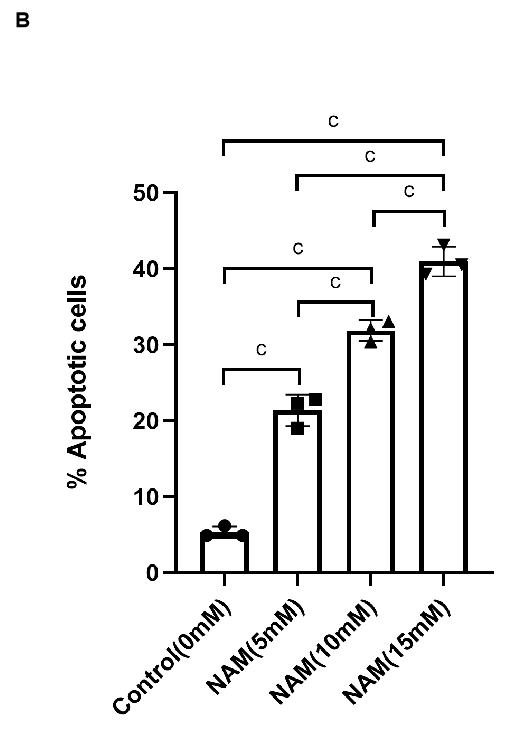
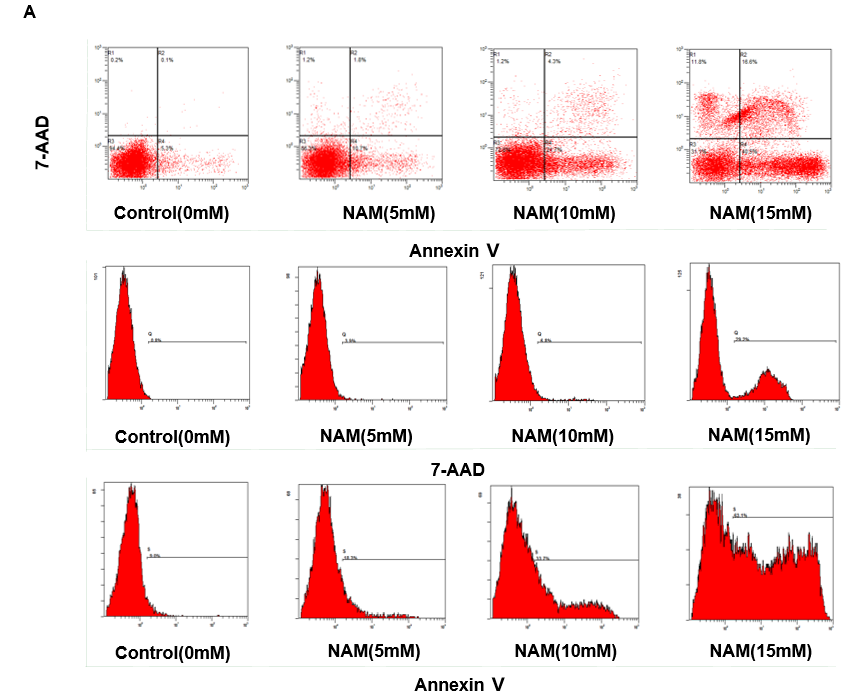
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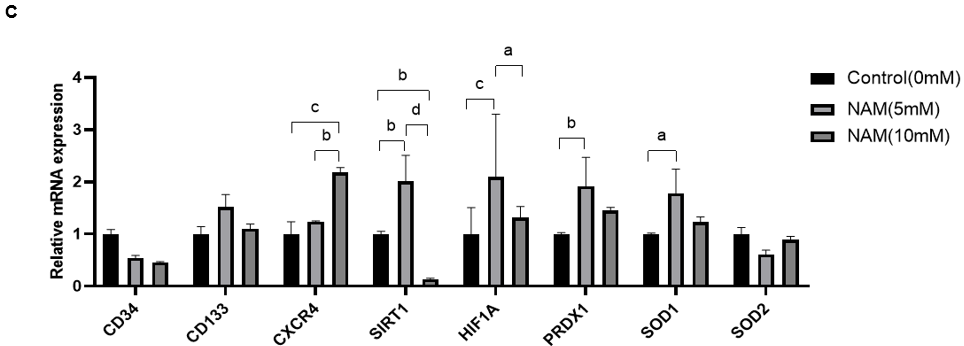
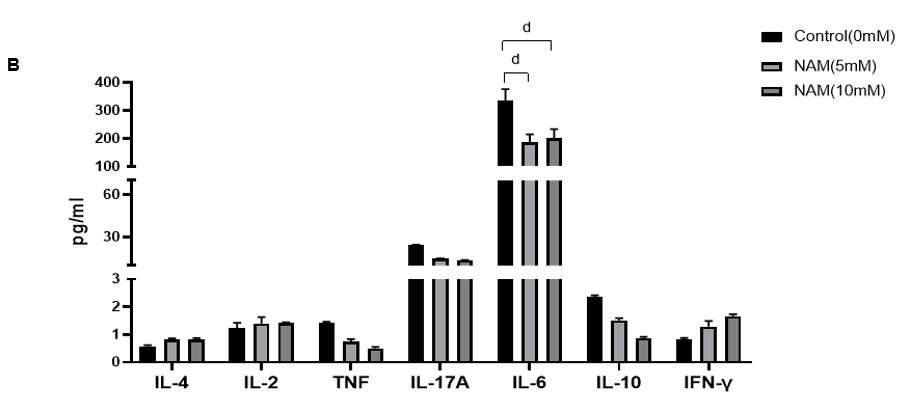
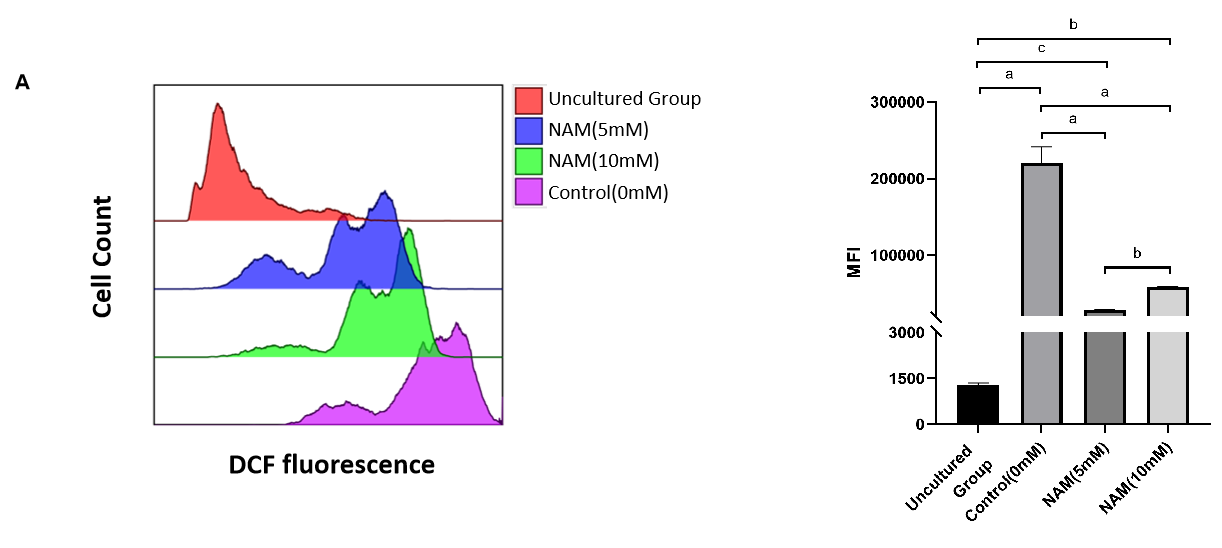
**Figure 1 Proportion of hematopoietic stem and progenitor cells, short-term** **repopulating** **hematopoietic stem cells and long-term repopulating hematopoietic stem cells populations in each group of cultured cells.** A: Phenotype (up) and proportion (down) of hematopoietic stem and progenitor cells; B: Phenotype (up) and proportion (down) of short-term repopulating hematopoietic stem cells (ST-HSCs); C: Phenotype (up) and proportion (down) of long-term repopulating HSCs; D: Cell size for each group of cells; E: Viability of each group of cells; F: Typical images of representative morphologies of various type of colonies as indicated; G: Colony formation assay showing the number of various types of cell colonies as indicated. The number of colony-forming unit (CFU)-erythroid, CFU-macrophage and CFU-granulocyte/erythroid/macrophage/megakaryocyte were scored. a*P* <0.05; b*P* < 0.01; c*P* < 0.001; d*P* < 0.0001; ns: Not significant. NAM: Nicotinamide; CFU-E: Colony-forming unit-erythroid; CFU-GM: Colony-forming unit-granulocyte, macrophage; CFU-GEMM: Colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte.



**Figure 2 Expansion efficiency of subsets of hematopoietic stem cells in different concentrations of nicotinamide.** A: Microscopic observation of cultured cells in each group (Bar = 200 μm); B: Fold expansion of total nucleated cells; C: Fold expansion of hematopoietic stem and progenitor cells; D: Fold expansion of short-term repopulating hematopoietic stem cells; E: Fold expansion of long-term repopulating hematopoietic stem cells. a*P* < 0.05; c*P* < 0.001; ns: Not significant. NAM: Nicotinamide; TNC: Total nucleated cells.



**Figure 3 Effect of different concentrations of nicotinamide on the proportion of apoptotic cells and cell cycle distribution.** A: Representative flow cytometry for apoptosis detection in each group; B: Proportion of apoptotic cells in each group; C: Comparison of cell cycle distribution among different groups. a*P* < 0.05; b*P* < 0.01; c*P* < 0.001. NAM: Nicotinamide.



**Figure 4** **Effect of different concentrations of nicotinamide on reactive oxygen species content, cytokine levels, and gene expression in cultured hematopoietic stem cells.** A: Reactive oxygen species in different groups of cells determined by mean fluorescence intensity; B: Cytokine levels in the supernatant of cultured cells in each group; C: Comparison of expression of genes encoding stemness-related factors, chemokines, components of hypoxia pathways, and antioxidant enzymes among different groups. a*P* < 0.05; b*P* < 0.01; c*P* < 0.001; d*P* < 0.0001. NAM: Nicotinamide; PRDX1: Peroxiredoxin 1; SOD: Superoxide dismutase; IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon.



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