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STEM CELLS TRANSLATIONAL MEDICINE

Coinhibition of activated p38 MAPKα and mTORC1 potentiates stemness maintenance of HSCs from SR1-expanded human cord blood CD34$^+$ cells via inhibition of senescence

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Abstract

The stemness of ex vivo expanded hematopoietic stem cells (HSCs) is usually compromised by current methods. To explore the failure mechanism of stemness maintenance of human HSCs, which were expanded from human umbilical cord blood (hUCB) CD34$^+$ cells, by differentiation inhibitor Stem Regenin 1 (SR1), an antagonist of aryl hydrocarbon receptor, we investigated the activity of p38 mitogen-activated protein kinase α (p38 MAPKα, p38α) and mammalian target of rapamycin complex 1 (mTORC1), and their effect on SR1-expanded hUCB CD34$^+$ cells. Our results showed that cellular senescence occurred in the SR1-expanded hUCB CD34$^+$ cells in which p38α and mTORC1 were successively activated. Furthermore, their coinhibition resulted in a further decrease in hUCB CD34$^+$ cell senescence without an effect on apoptosis, promoted the maintenance of expanded phenotypic HSCs without differentiation inhibition, increased the hematopoietic reconstitution ability of multiple lineages, and potentiated the long-term self-renewal capability of HSCs from SR1-expanded hUCB CD34$^+$ cells in NOD/Shi-scid/IL-2Rγ^{null} mice. Our mechanistic study revealed that senescence inhibition by our strategy was mainly attributed to the

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downregulation of the splicesome, proteasome formation, and pyrimidine metabolism signaling pathways. These results suggest that coinhibition of activated p38α and mTORC1 potentiates stemness maintenance of HSCs from SR1-expanded hUCB CD34$^+$ cells via senescence inhibition. Thus, we established a new strategy to maintain the stemness of ex vivo differentiation inhibitor-expanded human HSCs via coinhibition of multiple independent senescence initiating signal pathways. This senescence inhibition-induced stemness maintenance of ex vivo expanded HSCs could also have an important role in other HSC expansion systems.

KEYWORDS

cellular senescence, ex vivo expansion, HSC stemness maintenance, human cord blood CD34$^+$ cells, mammalian target of rapamycin complex 1, p38 mitogen-activated protein kinase α, Stem Regenin 1

1 | INTRODUCTION

Hematopoietic stem cell (HSC) transplantation is a curative therapy for many diseases.[1,2] However, the broad use of HSCs is impeded by limited cell quantity, especially for cord blood HSC transplantation.[3-5] Although HSCs can be generated from embryonic stem cells[6] or induced pluripotent stem cells[7] in vitro, the low efficiency and poor transplantability of the generated HSCs remain challenges.[2,5,8] Therefore, HSC expansion remains to be improved.

Historically, HSCs have been expanded in vitro in suspension cultures with medium supplemented with hematopoietic growth factors (HGFs), but the system led to HSC exhaustion rather than expansion by initiating differentiation and apoptosis.[4] Although tremendous efforts have been dedicated to improve the system, including blocking differentiation and promoting self-renewal,[9-15] or coculturing with niche cells to mimic the in vivo hematopoietic microenvironment,[1,2,4,5,8] the ex vivo expansion of human HSCs with long-term engraftment ability has remained unsuccessful. Therefore, unknown mechanisms involved in stemness maintenance in ex vivo expanded HSCs need to be unraveled.

Senescence recapitulates the aging process at the cellular level. Senescent cells exhibits permanent cell cycle arrest and a senescence-associated secretory phenotype.[16] Recent discoveries revealed that the senescence of stem cell populations during the aging process induces somatic stem cell exhaustion.[17,18] Thus, we speculated that the loss of stemness in ex vivo expanded HSCs could partly be attributed to the senescence program. Our studies first confirmed that senescence in ex vivo expanded HSCs was induced by activated p38 mitogen-activated protein kinase α (p38 MAPKα, p38α) or mammalian target of rapamycin complex 1 (mTORC1) and that these mechanisms compromised the long-term engraftment ability of the HSCs,[18-21] indicating that inhibition of cellular senescence might be a promising strategy for retaining stemness in ex vivo expanded HSCs.

p38α plays an important causative role in the senescence of cells following telomere shortening and environmental stress.[22] In contrast, mTORC1 induces cellular senescence mainly through the regulation of metabolic processes in response to nutrients, growth cues, and cellular energy status.[23] However, it remains to be investigated whether coinhibition of activated p38α and mTORC1 can further maintain stemness in ex vivo expanded human HSCs. Recently, it was shown that Stem Regenin 1 (SR1), an antagonist of aryl hydrocarbon receptor (AHR), could promote ex vivo expansion of hematopoietic stem and progenitor cells (HSPCs) via differentiation inhibition.[9] Thus, we questioned whether senescence occurs, whether both p38α and mTORC1 are activated in SR1-expanded human umbilical cord blood (hUCB) CD34$^+$ HSPCs, and whether the combined inhibition of both differentiation and senescence can further promote the expansion of HSCs with long-term hematopoietic reconstitution ability. Here, our results suggest that both p38α and mTORC1 are activated in SR1-expanded hUCB CD34$^+$ cells, and their coinhibition maintains the

Significance statement

The stemness of ex vivo-expanded hematopoietic stem cells (HSCs) is usually compromised by current methods. It is supposed that none of these methods could avoid senescence-associated stemness loss because HSC hyperproliferation and ex vivo culture microenvironments different from the real in vivo hematopoietic niche will induce expanded HSC senescence. Here, it was found that both p38α and mammalian target of rapamycin complex 1 are activated in differentiation inhibitor Stem Regenin 1 (SR1)-expanded human umbilical cord blood (hUCB) CD34$^+$cells. Their coinhibition maintains the stemness of HSCs from SR1-expanded hUCB CD34$^+$ cells through senescence inhibition mainly via downregulation of the splicesome, proteasome formation, and pyrimidine metabolism signaling pathways. This multiple senescence initiating signalings inhibition-induced stemness maintenance of ex vivo expanded HSCs could also play an important role in other HSC expansion systems.

stemness of HSCs from SR1-expanded hUCB $CD34^+$ cells through senescence inhibition mainly via downregulation of the splicesome, proteasome formation, and pyrimidine metabolism signaling pathways.

2 | MATERIALS AND METHODS

2.1 | Isolation and expansion of hUCB $CD34^+$ cells

hUCB was obtained from healthy puerperas in the Obstetrics Department of Tongji Hospital (Wuhan, China) with patient consent and approval from the medical ethics committee of Huazhong University of Science and Technology (HUST). The hUCB-derived mononuclear cells were separated through density gradient centrifugation, and then hUCB $CD34^+$ cells were enriched using a CD34 MicroBead Kit (Miltenyi Biotec, Germany) according to the manufacturer's protocol. $CD34^+$ cells were resuspended in StemSpan Serum-Free Expansion Medium (SFEM) (StemCell Technologies, Canada) supplemented with 100 ng/mL thrombopoietin, stem cell factor, Flt3 ligand, and IL-6 (PeproTech). Next, the cells were seeded in 12-well plates at a density of 3×10^4 cells/well, cultured in a humidified atmosphere with 5% CO_2 at 37°C, and treated with 200 nM LY2228820 (LY) (Selleck) or/and 1 μM SR1 (Calbiochem, Germany) on day 0 to inhibit activated p38α or/and AHR, respectively, and with 0.5 mL SFEM supplemented with 5 ng/mL rapamycin (Rapa) (Calbiochem) to inhibit overactivated mTORC1 on day 4. Half of the medium was renewed every 3 days along with the corresponding inhibitors and fresh HGFs. The cultured cells were collected on day 8 for subsequent studies.

2.2 | Detection of cell phenotypes

To determine the total number of living cells, cells were stained with 0.4% trypan blue (Beyotime, China) and counted under an inverted microscope. To determine the phenotypic HSPCs in culture, the cells were stained with the following antibodies: FITC-anti-human CD34, PE-anti-human CD38, APC-anti-human CD90, and PE-Cy7-anti-human CD45RA at 4°C for 30 minutes. To analyze the engraftment of human cells in mice, cells isolated from the blood, bone marrow, or spleens of mice were stained with APC-anti-human CD45, FITC-anti-mouse CD45, FITC- or APC-Cy7-anti-human CD3, PE-Cy7-anti-human CD19, PE-anti-human CD11b, PE-Cy5-anti-human CD33, PE-anti-human Gly-A(CD235a), PE-Cy7-anti-human CD56, PE-Cy5-anti-human CD41, APC-cy7-anti-human CD34, PE-anti-human CD38, PE-Cy5-anti-human CD90, or PE-Cy7-anti-human CD45RA antibodies at 4°C for 30 minutes. Cell phenotypes were evaluated by flow cytometry using an LSR II flow cytometer. All antibodies were purchased from Biolegend.

2.3 | Cell proliferation assay

The isolated hUCB $CD34^+$ cells were stained with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma) and cultured ex vivo for 7 days following the aforementioned protocol. On day 8, these cultured cells were collected and stained with PE-anti-human CD34 antibody, resuspended in 2% FBS-PBS, and then analyzed by flow cytometry.

2.4 | Detection of the cell cycle

Cultured cells were collected and $CD34^+$ cells were enriched using a CD34 MicroBead Kit. Next, $CD34^+$ cells were treated with 75% cold ethyl alcohol overnight at 4°C. The next day, the cells were incubated with PBS supplemented with 50 μg/mL propidium iodide (PI) (Sigma), 100 μg/mL RNase A (Beyotime), and 0.2% Triton X-100 (Beyotime) at 4°C for 30 minutes and then analyzed by flow cytometry.

2.5 | Detection of cell senescence

Cultured cells were collected and stained with PE-anti-human CD34 antibody. Next, the cells were incubated with prewarmed 33 μM C_{12}FDG (Invitrogen) at 37°C for 30 minutes. The fluorescence intensity was determined by flow cytometry.

2.6 | Detection of cell apoptosis

Cultured cells were collected and stained with PE-Cy5-anti-human CD34 antibody. Next, the cells were incubated with Annexin V-FITC and PI (BioVision) for 15 minutes in the dark at room temperature and then analyzed by flow cytometry.

2.7 | Test of colony forming units (CFUs)

Cultured cells were collected, enumerated, and suspended at a density of 1×10^4 cells/mL with Iscove's modified Dulbecco's medium (Gibco) supplemented with 2% FBS. Then, CFUs from the progeny of these cultured hUCB $CD34^+$ cells were detected following the manufacturer's protocol (StemCell Technologies).

2.8 | Determination of signal pathway activation

To determine the activation of p38α and mTORC1 in cultured cells, we analyzed the expression of p-P38, p-P70 S6, and p-4EBP1 by Western blotting. Cell lysate preparation and Western blotting were performed following our previous description.[24] To examine the effect of inhibitors of the p38α, mTORC1, and AHR signaling pathways, freshly isolated $CD34^+$ cells or their expanded progeny were first stained with PE-anti-human CD34 antibody and then fixed with intracellular fixation buffer (eBioscience) for 45 minutes, followed by treatment with permeabilization buffer (eBioscience) according to the recommended protocol. Next, the cells were incubated with primary

antibodies against p-P38 (Cell Signaling Technology), p-S6 (Cell Signaling Technology), or CYP1B1 (Abcam, UK) overnight at 4°C. The next day, the cells were incubated with Alexa Fluor 488-linked secondary antibodies (Invitrogen) at room temperature for 1 hour and then analyzed by flow cytometry.

2.9 | Engraftment of hUCB CD34$^+$ cells in NOD/Shi-*scid*/IL-2Rγ^{null} (NOG) mice

Six- to 10-week-old female NOG mice weighing 16 to 19 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China) and maintained in the Laboratory Animal Center of Wuhan University (China). The animal experiments were conducted in accordance with the ethical guidelines of the Animal Experiment Committee of HUST. For short-term hematopoietic reconstitution assays, 1000, 3000, 10 000, and 30 000 uncultured hUCB CD34$^+$ cells or a fraction of the final culture equivalent to 1000, 3000, and 10 000 starting hUCB CD34$^+$ cells were transplanted into sublethally irradiated (240 cGy) NOG mice via the tail vein within 24 hours after irradiation. The chimerism of donor cells in blood was analyzed by flow cytometry at 1, 4, and 8 weeks after transplantation. At 13 weeks posttransplantation, the mice were sacrificed, and the spleen and bone marrow of both femurs and tibiae were dissected. The chimerism of donor cells in the bone marrow and the spleen, the chimerism of human B cells in the spleen and human B cells, T cells, myeloid cells, megakaryocytes, red blood cell progenitors, natural killer (NK) cells, and phenotypic HSPCs in the recipient bone marrow were determined by flow cytometry. The hematopoietic reconstitution experiments were repeated four times using different batches of NOG mice and different sources of hUCB cells.

2.10 | Limiting dilution analysis

To calculate the NOG mouse-repopulating cell (SRC) frequency, 1000, 3000, and 5000 uncultured hUCB CD34$^+$ cells or a fraction of the final culture equivalent to 1000, 3000, and 5000 starting hUCB CD34$^+$ cells were transplanted into sublethally irradiated (230 cGy) NOG mice via the tail vein within 24 hours of irradiation. After 16 weeks, the mice were sacrificed, and the engraftment of donor cells in bone marrow was determined by flow cytometry. For secondary engraftment, half of the bone marrow cells of both femurs and tibiae from primary recipient mice were collected and transplanted into sublethally irradiated secondary recipient NOG mice for another 16 weeks. Then, the bone marrow cells of both femurs and tibiae were harvested from the secondary recipient mice, and engraftment of donor cells was analyzed by flow cytometry. Positive engraftment was determined by the presence of greater than 0.1% hCD45$^+$ mCD45$^-$ cells in recipient bone marrow. Poisson statistics were used to calculate SRC frequency. SRC frequency and significant differences between various groups were determined using L-Calc software (StemCell Technologies).

2.11 | Measurement of mitochondrial membrane potential, mass and reactive oxygen species production in hUCB CD34$^+$ cells

Freshly isolated hUCB CD34$^+$ cells or their expanded progeny were first incubated with JC-1 (5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (Invitrogen) or MitoTracker Deep Red FM (Invitrogen) or MitoSOX Red (Invitrogen) following the manufacturer's protocols followed by staining at 4°C with PE-Cy7-anti-human CD34 antibody (Biolegend) for 30 minutes and analysis by flow cytometry.

2.12 | Determination of telomere length in hUCB CD34$^+$ cells

The mean telomere length of uncultured CD34$^+$ cells or CD34$^+$ cells derived from expanded progeny of hUCB CD34$^+$ cells was determined by qPCR based on a modification of a previously described method.[25]

2.13 | Analysis of LC3B immunofluorescence

Freshly isolated CD34$^+$ cells or their expanded progeny was fixed with 4% paraformaldehyde. Then, the slides were treated with 0.5% Triton X-100 (Beyotime) at room temperature for 20 minutes. Next, the cells were blocked with goat serum (Boster, China) at room temperature for 30 minutes and incubated with rat anti-human LC3B antibody (Abcam) overnight at 4°C. The cells were next incubated with goat anti-rat antibody labeled with FITC (Boster) for 1 hour in the dark at room temperature. Nuclei were then stained with DAPI (Beyotime) in the dark for 5 minutes. Finally, images were captured under a confocal microscope (Nikon Corp, Japan) at ×600 magnification.

2.14 | Transcriptome sequencing

Uncultured and HGFs along with 0.1% DMSO (HGFs) or LY plus Rapa (LR) or SR1-treated hUCB CD34$^+$ cells for 4 days were lysed. Total RNA was extracted using TRIzol reagent (Takara, Japan). The transcriptome was analyzed by Annoroad Co. (Beijing, China). Normalization and comparisons between two sample groups were performed using DESeq2 (HGFs vs Uncultured, SR1 vs HGFs, LR vs HGFs). Our RNA-sequencing data were then treated using the Network Essentiality Scoring Tool (NEST) to precisely evaluate the genes expression differences.[26] For the NEST method, we collected gene-gene interactions from the STRING database (STRING v10.5) corresponding to evidence from the expression levels of interacting genes in the biological network. The original interaction score was normalized to the range of 0 to 1 with the max-min method and further used to calculate the NEST score. The fold-change value of each gene was fed into NEST to obtain the gene's final score. The transcriptome data are available in GEO: GSE151964.

2.15 | Enrichment analysis

Gene set enrichment analysis (GSEA) was performed using GSEA software with parameters set to 1000 gene-set permutations and gene-set sizes between 15 and 500. A ranked list of genes using the gene NEST value was performed for GSEA.[27] The updated MsigDB-hallmark, C2 and C5 gene set databases were used to identify pathways closely associated with LR or SR1 treatment. The enriched gene sets with FDR q-value <0.25 and NOM *P*-value <.05 were significant.[28]

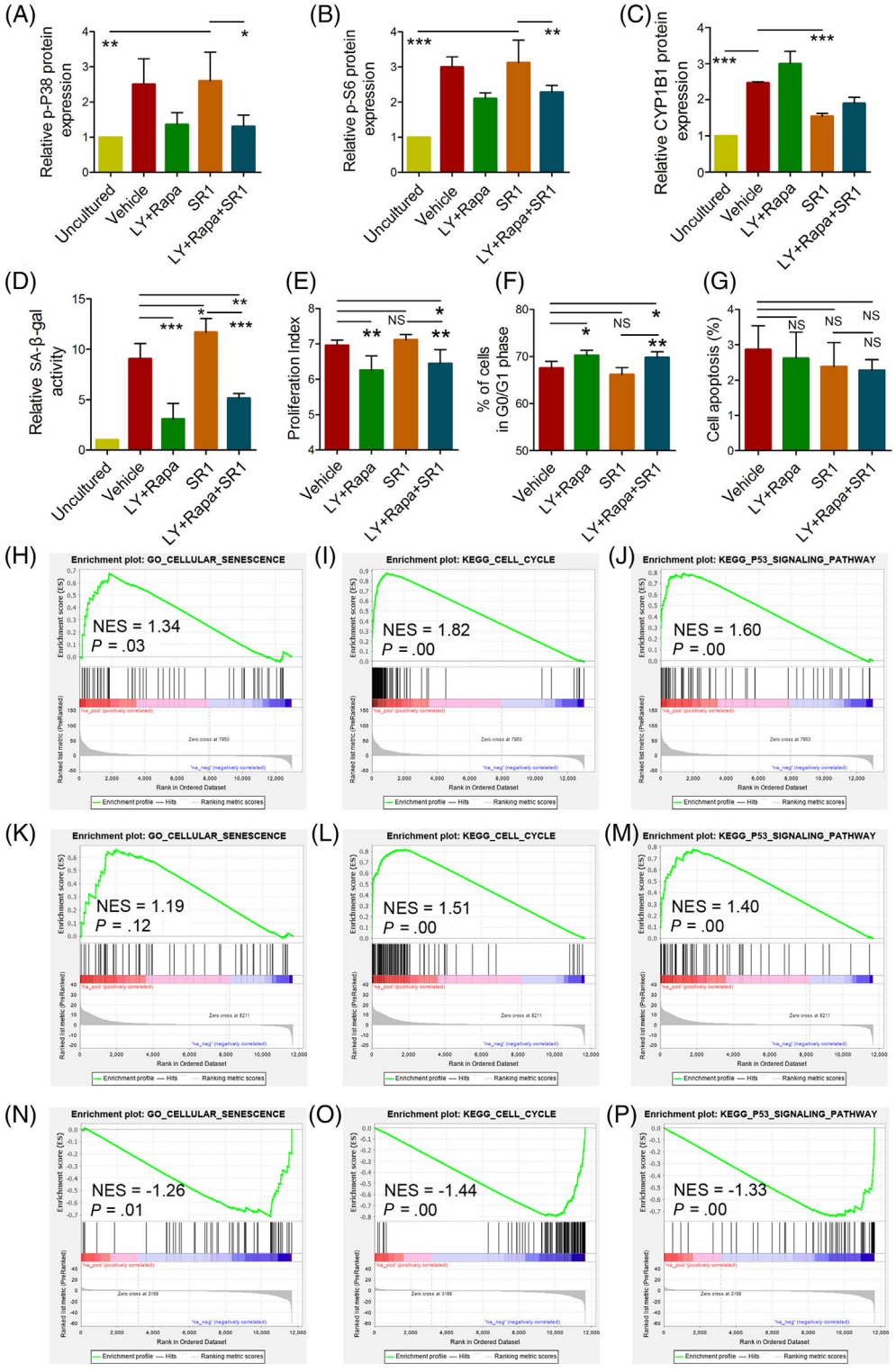

FIGURE 1 Legend on next page.

2.16 | Statistical analysis

All flow cytometry data were analyzed by FlowJo 7.6 software (Tree Star). Data are presented as the mean ± SD of at least three independent experiments. For engraftment, each data point represents an independent mouse, and the short line for each data set represents the mean engraftment. Statistical analyses were performed with SPSS using one-way analysis of variance, followed by the least significant difference test. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Coinhibition of activated p38α and mTORC1 prevents SR1-expanded hUCB CD34$^+$ cells from undergoing senescence without affecting apoptosis

First, we examined whether p38α, mTORC1, and AHR signaling were activated in ex vivo expanded hUCB CD34$^+$ cells treated with different methods for 7 days. Our data showed that p-p38α (a downstream molecule of p38α[29]), p-S6 (a downstream molecule of mTORC1 signaling[30]), and CYP1B1 (a downstream molecule of AHR signaling[31]) protein expression in HGF-expanded hUCB CD34$^+$ cells were significantly increased (Figure 1A-C; Figure S1). Interestingly, the expressions of p38α and p-S6 in SR1-expanded hUCB CD34$^+$ cells was also significantly higher than that in uncultured hUCB CD34$^+$ cells (Figure 1A,B). The increased expression of these activated proteins in HGFs and SR1-expanded hUCB CD34$^+$ cells could be downregulated by their respective inhibitors (LY, Rapa, or SR1) (Figure 1A-C; Figure S2). These results suggest that p38α, mTORC1, and AHR signalings are activated in ex vivo HGF-expanded hUCB CD34$^+$ cells and that p38α and mTORC1 are also activated in ex vivo SR1-expanded hUCB CD34$^+$ cells.

Further investigation revealed that senescence-associated β-galactosidase (SA-β-gal), a classical marker of senescent cells,[32] in HGF or SR1-expanded hUCB CD34$^+$ cells exhibited a 9.06- or 11.70-fold increase relative to the activity in uncultured hUCB CD34$^+$ cells (Figure 1D). Interestingly, coinhibition of activated p38α and mTORC1 resulted in 43.07% or 43.49% downregulation of SA-β-gal activity relative to that in cells with separate inhibition of p38α or mTORC1 (Figure S3A). More importantly, coinhibition of activated p38α and mTORC1 along with SR1 induced a 55.90% decrease in SA-β-gal activity compared with that in SR1-treated hUCB CD34$^+$ cells (Figure 1D). We also found that coinhibition of activated p38α and mTORC1 inhibited proliferation and increased the proportion of G0/G1 phase cells without affecting apoptosis in SR1- or HGF-expanded hUCB CD34$^+$ cells (Figure 1E-G; Figure S3B-D). These results suggest that coinhibition of activated p38α and mTORC1 could further prevent SR1- or HGF-expanded hUCB CD34$^+$ cells from undergoing cellular senescence and help maintain them in the G0/G1 phase of the cell cycle. Furthermore, our transcriptome data showed that cellular senescence-associated signaling pathways, including cellular senescence, cell cycle, and p53 signaling pathways,[33,34] were upregulated in HGF-expanded hUCB CD34$^+$ cells compared with uncultured hUCB CD34$^+$ cells (Figure 1H-J). Furthermore, both the cell cycle and p53 signaling pathways were further upregulated in SR1-expanded hUCB CD34$^+$ cells compared with HGF-expanded hUCB CD34$^+$ cells (Figure 1L,M). However, coinhibition of activated p38α and mTORC1 downregulated these cellular senescence-associated signaling pathways compared with these pathways in HGF-expanded hUCB CD34$^+$ cells (Figure 1N-P). These results further support our hypothesis that coinhibition of activated p38α and mTORC1 prevents SR1-expanded hUCB CD34$^+$ cells from undergoing senescence without affecting apoptosis.

3.2 | Coinhibition of activated p38α and mTORC1 results in an increased maintenance of phenotypic HSCs without differential inhibition in ex vivo SR1-expanded hUCB CD34$^+$ cells

Since coinhibition of activated p38α and mTORC1 further prevented HGF-expanded hUCB CD34$^+$ cells from undergoing senescence compared with inhibition of activated p38α or mTORC1 signaling separately, we questioned whether coinhibition of activated p38α and mTORC1 could further promote HSC expansion. Our results showed

FIGURE 1 Coinhibition of activated p38α and mTORC1 prevents SR1-expanded hUCB CD34$^+$ cells from undergoing senescence without affecting apoptosis. hUCB CD34$^+$ cells were cultured ex vivo for 7 days with only HGFs along with 0.1% DMSO (vehicle) or/and SR1 or/and inhibitors of p38α (LY) and mTORC1 (Rapa). The expression of p-P38 (A) (n = 3), p-S6 (B) (n = 3), CYP1B1 (C) (n = 4), and SA-β-gal activity (D) detected using C12FDG staining (n = 3) in uncultured and expanded CD34$^+$ cells were determined by flow cytometry. The proliferation (E) of hUCB CD34$^+$ cells was determined based on CFSE staining and flow cytometry (n = 4). The cell cycle distribution (F) of hUCB CD34$^+$ cells was determined based on PI staining and flow cytometry (n = 4). The cell apoptosis (G) of hUCB CD34$^+$ cells was analyzed by Annexin V and PI staining and flow cytometry (n = 4). For transcriptome sequencing and gene set enrichment analysis (GSEA), the hUCB CD34$^+$ cells of unculture or culture with HGFs or HGFs plus SR1 (SR1) or HGFs plus both p38α and mTORC1 inhibitors (LR) treatment for 4 days were collected, their transcriptome sequences were detected and the gene NEST score based on the expression level of interacting genes in the biological network was determined using the NEST method to precisely evaluate the differences in gene expression among these different groups. Next, the GSEA was carried out. The GSEA data for cellular senescence, cell cycle, and p53 pathways from the comparison between HGF-expanded hUCB CD34$^+$ cells and uncultured hUCB CD34$^+$ cells (H-J) or between SR1-expanded hUCB CD34$^+$ cells (K-M) or LR-expanded hUCB CD34$^+$ cells (N-P) and HGF-expanded hUCB CD34$^+$ cells are shown. ***$P < .001$, **$P < .01$, and *$P < .05$; NS, no significance. CFSE, carboxyfluorescein diacetate succinimidyl ester; HGF, hematopoietic growth factor; hUCB, human umbilical cord blood; SR1, Stem Regenin 1

that coinhibition of activated p38α and mTORC1 further promoted maintenance but did not increase the expansion of hUCB-derived phenotypic HSPCs compared with inhibition of p38α or mTORC1 alone (Figures S4 and S5).

Since coinhibition of activated p38α and mTORC1 could lead to a marked decrease in the senescence of SR1-expanded hUCB $CD34^+$ cells and promoted the maintenance of ex vivo HGF-expanded hUCB-derived phenotypic HSCs, we next questioned whether coinhibition of activated p38α and mTORC1 could promote the ex vivo expansion of HSCs from SR1-expanded hUCB $CD34^+$ cells. Our data showed that coinhibition of activated p38α and mTORC1 resulted in a substantial increase in the proportion of $CD34^+CD38^-$, $CD34^+CD90^+$, $CD34^+CD45RA^-$, and $CD34^+CD38^-CD90^+CD45RA^-$ subpopulations, which are enriched in HSCs and multilineage progenitor cells,[9] by 59.09%-160.38% and 42.72%-217.58% relative to coinhibition of p38α and mTORC1 or SR1 treatment, respectively (Figure 2A-D; Figure S6). This strategy also increased the fold expansion of $CD34^+CD38^-$, $CD34^+CD90^+$, $CD34^+CD45RA^-$, and $CD34^+CD38^-CD90^+CD45RA^-$ subpopulations by 75.90%-186.85% relative to coinhibition of p38α and mTORC1 (Figure 2E-H). Although the total number of HGFs or SR1-expanded hUCB $CD34^+$ cell progeny decreased (Figure S7), this strategy increased the fold expansion of the $CD34^+CD45RA^-$ subpopulation by 19.56%, the $CD34^+CD38^-CD90^+CD45RA^-$ subpopulation by 72.80%, and maintained the fold expansion of the $CD34^+CD90^+$ subpopulation relative to SR1 treatment alone. Furthermore, culture for only 7 days led to a 17.05-fold increase in the $CD34^+CD90^+$ subpopulation, a 7.09-fold increase in the $CD34^+CD45RA^-$ subpopulation, and 34.68-fold increase in the $CD34^+CD38^-CD90^+CD45RA^-$ subpopulation respectively, relative to uncultured hUCB $CD34^+$ cells (Figure 2E-H). However, this strategy did not increase the number of CFUs relative to SR1 treatment alone (Figure 2I), suggesting that HPCs did not undergo further expansion in the culture system. Thus, the coinhibition of activated p38α and mTORC1 mainly promotes the maintenance of ex vivo SR1-expanded phenotypic HSCs not via inhibition of differentiation, because the strategy did not result in a further significant increase in the absolute number of phenotypic HPCs compared with that in SR1 cultured group.

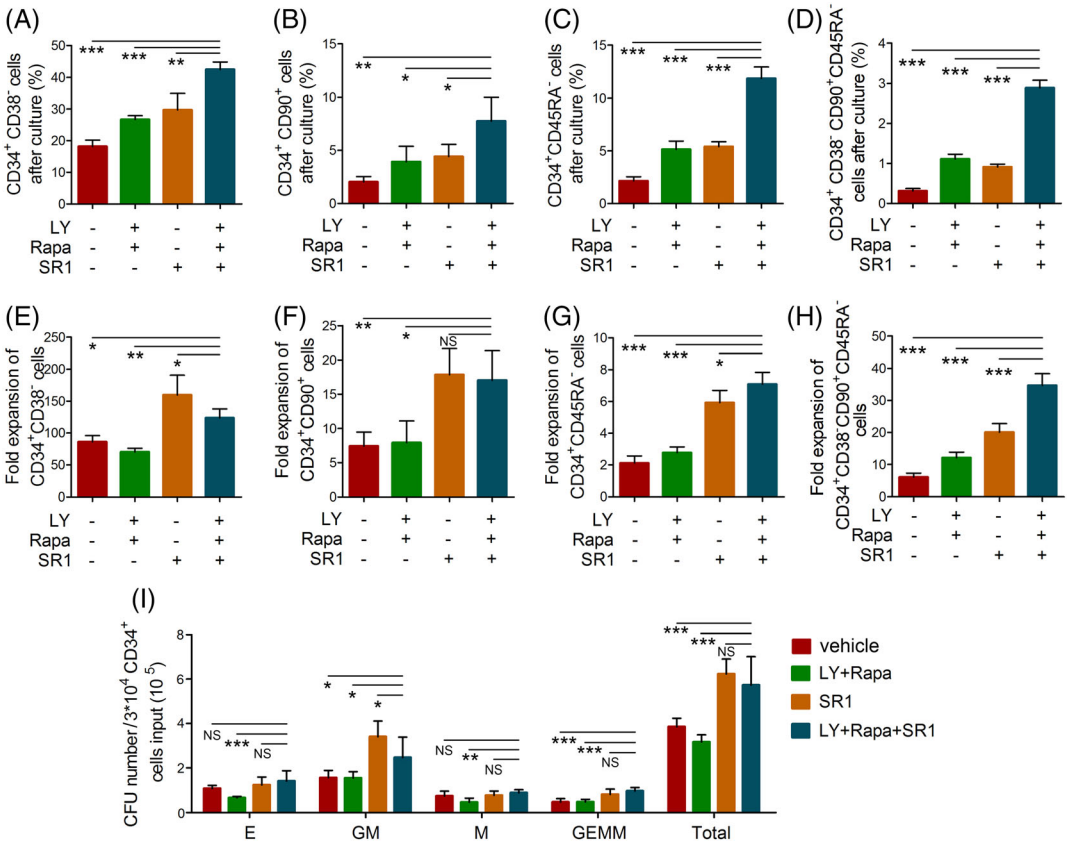

FIGURE 2 Coinhibition of activated p38α and mTORC1 promotes the maintenance of phenotypic HSCs without differentiation inhibition in ex vivo SR1-expanded hUCB $CD34^+$ cells. hUCB $CD34^+$ cells were cultured ex vivo for 7 days with only HGFs along with 0.1% DMSO (vehicle) or/and SR1 or/and LY and Rapa. The proportions (A-D) and fold expansions (E-H) of $CD34^+CD38^-$, $CD34^+CD90^+$, $CD34^+CD45RA^-$, and $CD34^+CD38^-CD90^+CD45RA^-$ subpopulations were determined by flow cytometry and manual calculation, respectively (n = 3). The numbers (I) of CFU-E, CFU-GM, CFU-M, CFU-GEMM and total CFUs produced by the cultured progeny of 3×10^4 hUCB $CD34^+$ cells under different conditions for 7 days (n = 6). The fold expansion of subpopulations was calculated by the following method: fold expansion = total cells × percentage of subpopulations after culture/input cells (uncultured hUCB $CD34^+$ cells) (3×10^4) × percentage of subpopulations before culture. ***$P < .001$, **$P < .01$, and *$P < .05$; NS, no significance. HGF, hematopoietic growth factor; hUCB, human umbilical cord blood; SR1, Stem Regenin 1

3.3 | Coinhibition of p38α and mTORC1 increases the ability of ex vivo SR1-expanded hUCB CD34$^+$ cells to produce multiple lineage hematopoietic reconstitution in vivo

To determine the hematopoietic reconstitution ability of the progeny of hUCB CD34$^+$ cells expanded with inhibitors of p38α, mTORC,1 and AHR, grafts of uncultured hUCB CD34$^+$ cells or their cultured progeny in sublethally irradiated NOG mice were evaluated. Our results showed that the progeny of 10 000 hUCB CD34$^+$ cells cultured with SR1 plus inhibitors of both p38α and mTORC1 for 7 days resulted in a significantly greater extent of donor cell engraftment in the peripheral blood of recipient mice at 8 weeks than the same number of uncultured hUCB CD34$^+$ cells (2.37-fold), the progeny of

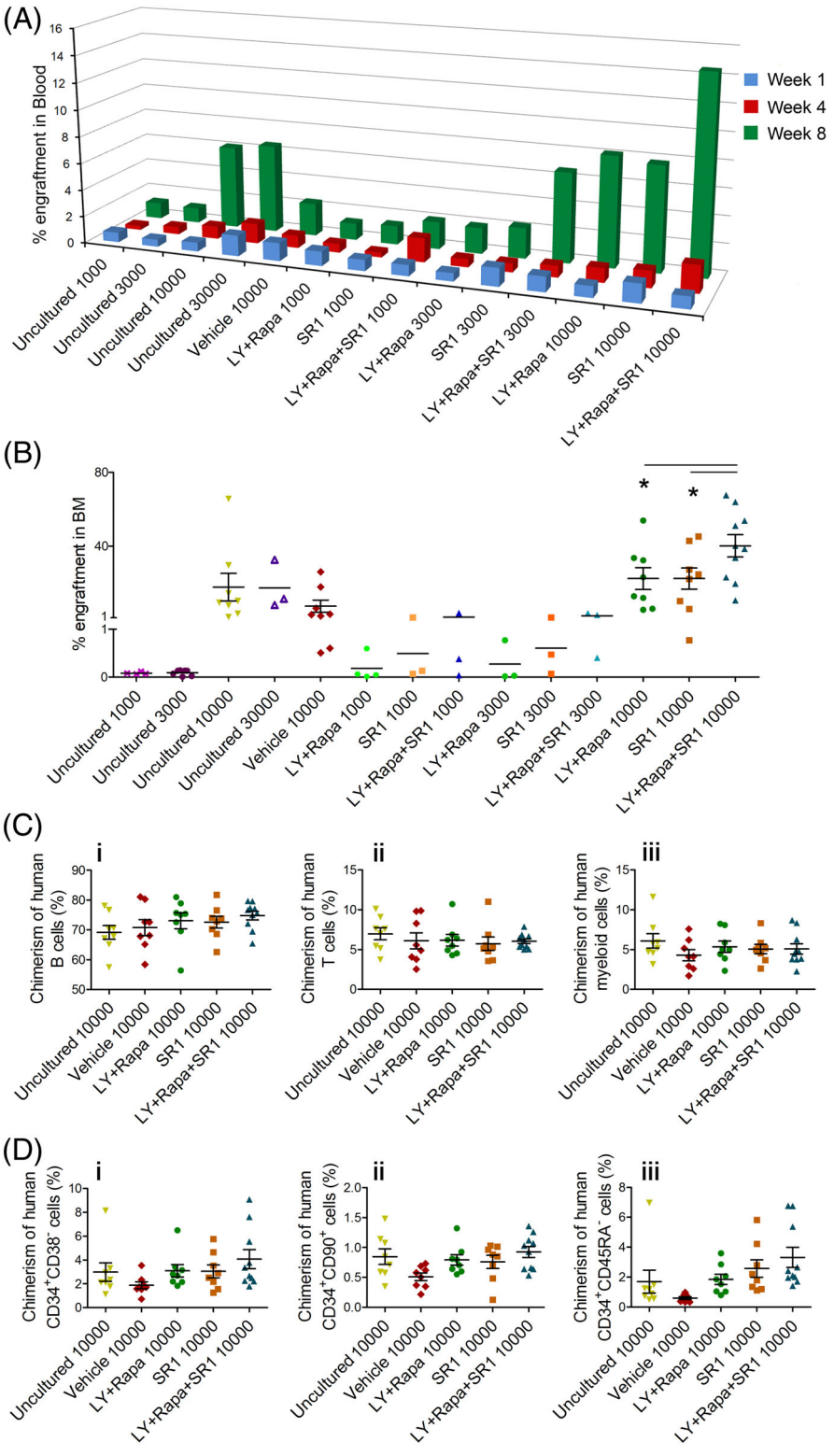

FIGURE 3 Coinhibition of activated p38α and mTORC1 increases the multiple lineages hematopoietic reconstitution ability and the in vivo maintenance of phenotype HSCs from ex vivo SR1 expanded hUCB CD34$^+$ cells. A total of 1000, 3000, 10 000, and 30 000 uncultured hUCB CD34$^+$ cells or a fraction of the final culture equivalent to 1000, 3000, and 10 000 starting hUCB CD34$^+$ cells cultured ex vivo for 7 days with only HGFs along with 0.1% DMSO (vehicle) or/and SR1 or/and LY and Rapa were transplanted into sublethally irradiated (240 cGy) 6-10-week-old female NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice via the tail vein within 24 hours after irradiation. The average percentage engraftment of human donor cells (hCD45$^+$mCD45$^-$) in the blood (A) at weeks 1, 4, and 8 and in the bone marrow (B) at week 13 are shown. The chimerism of human B cells (CD19$^+$CD3$^-$), T cells (CD3$^+$CD19$^-$), and myeloid cells (CD11b$^+$CD33$^+$) (C-i-iii), and the human CD34$^+$CD38$^-$, CD34$^+$CD90$^+$, and CD34$^+$CD45RA$^-$ subpopulations (D-i-iii) in the bone marrow at week 13 are shown. Each data point represents an individual mouse, and the short line for each data set represents the mean engraftment. *P < .05. HGF, hematopoietic growth factor; hUCB, human umbilical cord blood; SR1, Stem Regenin 1

10 000 hUCB CD34$^+$ cells cultured with vehicle (6.00-fold), the progeny of 10 000 hUCB CD34$^+$ cells cultured with inhibitors of p38α and mTORC1 (1.78-fold), or the progeny of 10 000 hUCB CD34$^+$ cells cultured with SR1 (1.87-fold) (Figure 3A; Figure S8). Furthermore, the progeny of 10 000 hUCB CD34$^+$ cells cultured with SR1 and inhibitors of both p38α and mTORC1 for 7 days led to greater donor cell engraftment of mouse bone marrow than the same number of uncultured hUCB CD34$^+$ cells (2.71-fold), the progeny of 10 000 hUCB CD34$^+$ cells cultured with vehicle (7.51-fold), the progeny of 10 000 hUCB CD34$^+$ cells cultured with inhibitors of both p38α and mTORC1 (1.93-fold), or the progeny of 10 000 hUCB CD34$^+$ cells cultured with SR1 (2.01-fold). Similar results were obtained in studies with 1000 and 3000 uncultured hUCB CD34$^+$ cells and their cultured progeny (Figure 3B).

In addition, the multilineage differentiation potential for the progeny of hUCB CD34$^+$ cells cultured with SR1 plus inhibitors of p38α and mTORC1 was investigated. Our results showed that the bone marrow and spleens of graft recipient mice had similar number of human T and B cells, myeloid cells, red blood cell progenitors, megakaryocytes, and NK cells as uncultured hUCB CD34$^+$ cells (Figure 3C-i-iii; Figures S9-S11). More interestingly, hUCB CD34$^+$ cells cultured with SR1 and inhibitors of p38α and mTORC1 resulted in a 2.25- or 5.28-fold higher engraftment of human CD34$^+$CD45RA$^-$ subpopulations than uncultured or HGF-treated cells (Figure 3D-i-iii), indicating that phenotypic HSCs with multilineage hematopoietic reconstitution ability were also maintained in vivo.

3.4 | Coinhibition of activated p38α and mTORC1 increases the long-term self-renewal capability of HSCs from ex vivo SR1-expanded hUCB CD34$^+$ cells

Since we confirmed that coinhibition of activated p38α and mTORC1 increases the hematopoietic reconstitution ability of multiple lineages and the in vivo maintenance of HSCs for ex vivo SR1-expanded hUCB CD34$^+$ cells, we next studied whether this strategy could further promote stemness maintenance of HSCs with long-term self-renewal capability. Human cells capable of hematopoietic reconstitution in immune-deficient mice (NSG or NOG mice) are termed NSG or NOG mouse-repopulating cells (SRCs), which represent candidate human

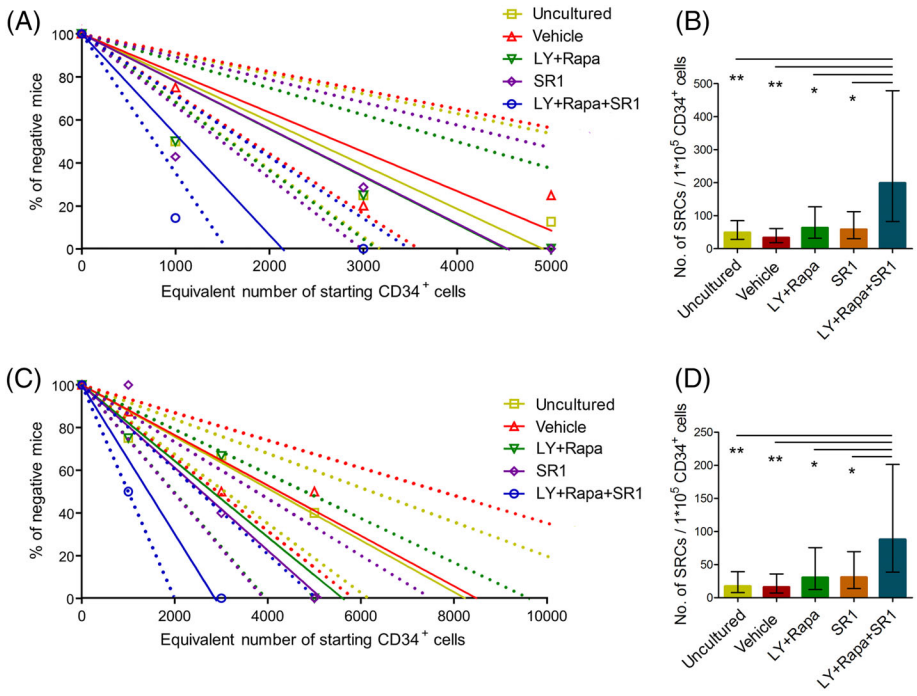

FIGURE 4 Coinhibition of activated p38α and mTORC1 increases the long-term self-renewal capability of HSCs from ex vivo SR1-expanded hUCB CD34$^+$ cells. A total of 1000, 3000, and 5000 uncultured hUCB CD34$^+$ cells or a fraction of the final culture equivalent to 1000, 3000, and 5000 starting hUCB CD34$^+$ cells cultured ex vivo for 7 days with only HGFs along with 0.1% DMSO (vehicle) or/and SR1 or/and LY and Rapa were transplanted into sublethally irradiated (230 cGy) NOG mice via the tail vein within 24 hours after irradiation. After 16 weeks, the mice were sacrificed, and the engraftment of donor cells in bone marrow was determined by flow cytometry. For secondary engraftment, half of the bone marrow cells of both femurs and tibiae from primary recipient mice were collected and transplanted into sublethally irradiated secondary recipient NOG mice for another 16 weeks. Then, the mice were sacrificed, and the engraftment of donor cells in bone marrow was determined by flow cytometry. Linear-regression for the percentage of negative mice and infused cell dose about primary (A) and secondary (C) transplantation. Solid lines represent the best-fit linear regression model for each data set. Dotted lines indicate the 95% confidence intervals. The different open shapes denote the percentage of negative animals for each dose of cells. Determination of the number of SRCs produced by 1×10^5 uncultured hUCB CD34$^+$ cells or the progeny of 1×10^5 hUCB CD34$^+$ cells expanded ex vivo in primary (B) and secondary (D) recipient mice. Data are presented as the means ±95% confidence intervals. $^{**}P < .01$ and $^{*}P < .05$. HGF, hematopoietic growth factor; hUCB, human umbilical cord blood; SR1, Stem Regenin 1

HSCs.[35] We transplanted 1000, 3000, and 5000 uncultured hUCB CD34$^+$ cells or a fraction of the final culture equivalent to 1000, 3000, and 5000 starting hUCB CD34$^+$ cells into sublethally irradiated NOG mice (Table S1; Figure S12A). The hUCB CD34$^+$ cells cultured with SR1 plus inhibitors of p38α and mTORC1 for 7 days resulted in a 4.06-, 5.96-, 3.14-, and 3.39-fold higher SRC frequency (1/503) than

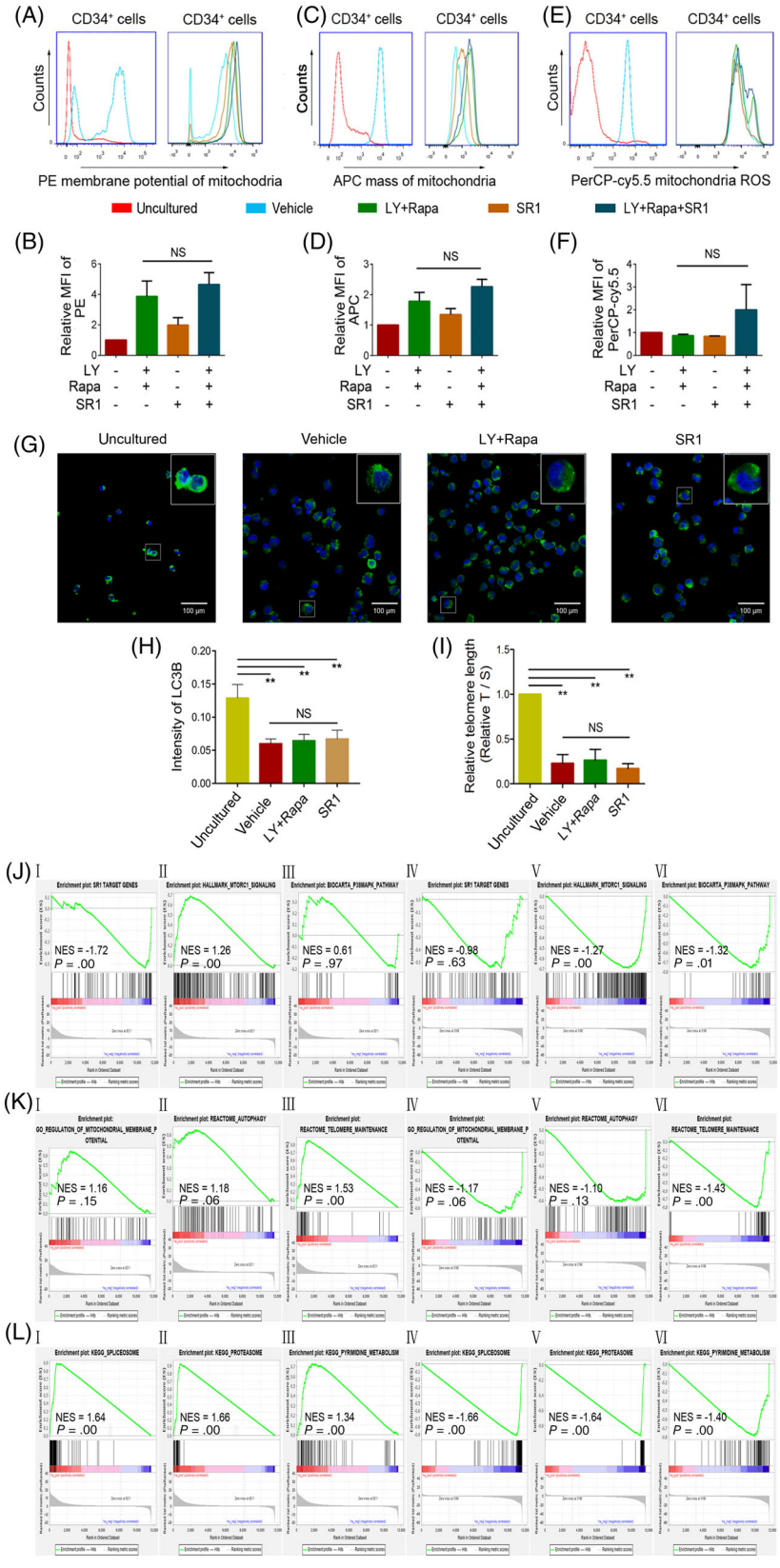

FIGURE 5 Coinhibition of activated p38α and mTORC1 prevents ex vivo expanded hUCB CD34$^+$ cells from undergoing senescence mainly via downregulation of the splicesome, proteasome formation, and pyrimidine metabolism signaling pathways. The hUCB CD34$^+$ cells were cultured ex vivo for 7 days with only HGFs along with 0.1% DMSO (vehicle) or HGFs plus SR1 (SR1) or HGFs plus coinhibition of activated p38α and mTORC1 (LY + Rapa). Then, the uncultured hUCB CD34$^+$ cells (uncultured) and the progeny of cultured hUCB CD34$^+$ cells were used for detection. Representative fluorescence line diagrams of mitochondrial membrane potential (A), mass (C), and reactive oxygen species (ROS) production (E) in these hUCB CD34$^+$ cells under different treatments. Quantitative analysis of the mean fluorescence intensity of mitochondrial membrane potential (B), mitochondrial mass (D), and mitochondrial ROS production (F) in hUCB CD34$^+$ cells cultured under various conditions relative to HGF-cultured hUCB CD34$^+$ cells (n = 5). The representative immunofluorescence for LC3B (green) and DAPI (blue) (G) of the uncultured hUCB CD34$^+$ cells or the progeny of cultured hUCB CD34$^+$ cells under different treatments. The percentage of these cells with autophagosomes indicated by quantification of LC3B-positive puncta (H) (n = 3). (I) The telomere lengths in these hUCB CD34$^+$ cells under different treatments were determined by qPCR (n = 3). For transcriptome sequencing and GSEA, the hUCB CD34$^+$ cells cultured with only HGFs, with HGFs plus SR1 (SR1) or with HGFs plus inhibitors of both p38α and mTORC1 (LR) for 4 days were collected, their transcriptome sequences were detected, and the gene NEST score based on the expression level of interacting genes in the biological network was determined using the NEST method. Next, the GSEA was carried out. The GSEA results of the signaling pathways of SR1 target genes, mTORC1 and p38α in SR1-cultured (J-I-III) and LR-cultured (J-IV-VI) hUCB CD34$^+$ cells relative to those in HGF-cultured hUCB CD34$^+$ cells. The GSEA results of the signaling pathways of mitochondrial membrane potential, autophagy and telomere maintenance in SR1-cultured (K-I-III) and LR-cultured (K-IV-VI) hUCB CD34$^+$ cells relative to those in HGF-cultured hUCB CD34$^+$ cells. The GSEA data for the splicesome, proteasome and pyrimidine metabolism signaling pathways in SR1-cultured (L-I-III) and LR-cultured (L-IV-VI) hUCB CD34$^+$ cells relative to those in HGF-cultured hUCB CD34$^+$ cells (n = 3).** $P < .01$. HGF, hematopoietic growth factor; hUCB, human umbilical cord blood; SR1, Stem Regenin 1

uncultured (1/2040), vehicle-treated (1/2998), LY and Rapa cotreated (1/1580), and SR1 treated (1/1707) hUCB CD34$^+$ cells, respectively (Figure 4A,B; Table S2).

To determine whether hUCB CD34$^+$ cells expanded with coinhibition of activated p38α and mTORC1 plus SR1 were capable of long-term engraftment, half of the bone marrow cells from primary recipient mice were injected into NOG secondary recipient mice (Table S1; Figure S12B). Poisson statistics revealed that hUCB CD34$^+$ cells cultured with SR1 plus inhibitors of p38α and mTORC1 resulted in a 5.08-, 5.54-, 2.89-, and 2.86-fold higher SRC frequency (1/1136) than uncultured (1/5776), vehicle-treated (1/6294), LY and Rapa cotreated (1/3283), and SR1-treated (1/3244) hUCB CD34$^+$ cells, respectively after another 16 weeks (Figure 4C,D; Table S3). If same number of bone marrow cells from each primary recipient mice were transplanted into secondary transplantation receipt mice, we might be able to obtain additional information to determine the secondary repopulation potential of individual hUCB CD34$^+$ cells or their cultured progeny from different treated groups.

3.5 | Coinhibition of activated p38α and mTORC1 prevents ex vivo expanded hUCB CD34$^+$ cells from undergoing senescence mainly via downregulation of the splicesome, proteasome formation, and pyrimidine metabolism signaling pathways

Since we confirmed that coinhibition of activated p38α and mTORC1 maintains the stemness of HSCs from ex vivo SR1-expanded hUCB CD34$^+$ cells via inhibition of senescence without affecting cellular apoptosis and differentiation, we next investigated the mechanisms of senescence downregulation in these expanded hUCB CD34$^+$ cells. First, we investigated their mitochondrial function, autophagy ability, and telomere length, which have been implicated in cellular senescence regulation.[36-39] Our results showed that coinhibition of p38α and mTORC1 and/or AHR signaling pathways neither decreased mitochondrial activity compared with that in HGF-expanded cells (Figure 5A-F) nor led to an increased number of autophagosomes or a longer telomere length than in HGF-cultured or uncultured cells (Figure 5G-I), suggesting that the downregulation of activated p38α and mTORC1 did not inhibit senescence through decreased mitochondrial activation or increased autophagy or maintenance of telomere length. These discoveries were further confirmed by our transcriptome study on these cultured hUCB CD34$^+$ cells, in which SR1 treatment seemed to upregulate the telomere maintaining signaling pathway, while LR treatment induced downregulation without the important changes of mitochondrial membrane potential and autophagy-associated signaling pathways compared with that in only the HGF culture group (Figure 5K-I-VI).

To further determine the molecular mechanisms by which these proteins downregulate senescence in ex vivo expanded hUCB CD34$^+$ cells by coinhibition of activated p38α and mTORC1, we performed RNA sequencing and transcriptome analysis in cultured hUCB CD34$^+$ cells with different treatments. Our transcriptome sequencing and GSEA data showed that SR1 treatment resulted in marked downregulation of its target genes but did not inhibit the activation of the p38α and mTORC1 signaling pathways related to HGF-expanded hUCB CD34$^+$ cells (Figure 5J-I-III). However, LR treatment led to notable downregulation of p38α and mTORC1 signaling related to HGF-expanded hUCB CD34$^+$ cells (Figure 5J-IV-VI). These findings further support our hypothesis that the activation of the p38α and mTORC1 signaling pathways occurs in SR1-expanded hUCB CD34$^+$ cells. Interestingly, we found that SR1 treatment led to upregulation of the spliceosome, proteasome, and pyrimidine metabolism signaling pathways, which have been confirmed to be implicated in cellular senescence regulation,[40-42] compared with HGF treatment (Figure 5L-I-III), but these pathways were significantly downregulated by LR treatment related to HGF treatment (Figure 5L-IV-VI), suggesting that coinhibition of the activated p38α and mTORC1 signaling pathways prevents ex vivo expanded hUCB CD34$^+$ cells from undergoing senescence mainly via downregulation of the spliceosome, proteasome formation, and pyrimidine metabolism signaling pathways.

4 | DISCUSSION

Many previous efforts have been dedicated to expand hUCB HSCs in vitro, including studies of SR1 via a differentiation block, pyrimidoindole derivative UM171 by increasing self-renewal, and antagonism of PPARγ signaling by enhancing glycolysis.[9,12,14] However, we posited that none of these methods of expanding HSCs could avoid senescence-associated stemness loss because HSC hyperproliferation and ex vivo culture microenvironments different from the real in vivo hematopoietic niche will induce expanded HSC senescence.

Our results suggest that senescence occurs in ex vivo differentiation inhibitor SR1-expanded hUCB CD34$^+$ cells in which p38α and mTORC1 are activated. Particularly, the coinhibition of activated p38α and mTORC1 prevents these cells from undergoing senescence to a greater extent than inhibition of activated p38α or mTORC1 separately, without decreasing apoptosis. This result was supported by decreased SA-β-gal activity, an increased number of cells with G0/G1 phase, proliferation inhibition, and downregulation of senescence-associated signaling pathways, including cellular senescence, cell cycle, and p53 signaling pathways, in our ex vivo SR1-expanded hUCB CD34$^+$ cells.

Our results further reveal that although coinhibition of activated p38α and mTORC1 does not result in a marked increase in HPC number from SR1-expanded hUCB CD34$^+$ cells, it leads to significant maintenance of the phenotype HSC proportion without blocking differentiation compared with SR1-expanded hUCB CD34$^+$ cells. More importantly, our functional research in immune-deficient mice on HSCs showed that this strategy also promotes stemness maintenance of HSCs from SR1-expanded hUCB CD34$^+$ cells, as coinhibition of activated p38α and mTORC1 not only potentiated multilineage hematopoietic reconstitution ability and the maintenance of in vivo cell numbers but also promoted the long-term self-renewal capability of HSCs from SR1-expanded hUCB CD34$^+$ cells.

Although coinhibition of activated p38α and mTORC1 along with SR1 did not result in an importantly increased phenotypic HSPCs expansion folds related to SR1-expanded hUCB $CD34^+$ cells, but only 7 days of culture in this system led to a 17.05-, 7.09-, and 34.68-fold increase in the $CD34^+CD90^+$, the $CD34^+CD45RA^-$, and the $CD34^+CD38^-CD90^+CD45RA^-$ subpopulation, respectively, relative to that in input uncultured cells. These results suggest that our system can effectively promote the expansion of HSCs with stemness maintenance of hUCB $CD34^+$ cells. Although 7-day culture using our system only led to 5.08-, 5.54-, 2.89-, and 2.86-fold higher SRC frequency than uncultured, HGF-treated, LY and Rapa cotreated, and SR1-treated hUCB $CD34^+$ cells, respectively, in the second 16-week graft, two- to three-fold ex vivo expansion of HSCs with normal stemness would have a profound clinical impact according to clinical studies of cord blood grafts from different donors.[43] In addition, we may obtain more HSCs than other groups[9,12,14] if the expansion time is extended beyond 7 days, although a shorter expansion process is more suitable for clinical application owing to the concern of safety and reduced treatment time for patients.

Furthermore, our mechanistic study suggests that coinhibition of activated p38α and mTORC1 promotes the stemness maintenance of HSCs from SR1-expanded hUCB $CD34^+$ cells via cellular senescence inhibition, as downregulated senescence and inhibited hyperproliferation were observed without decreased apoptosis or differentiation block. Interestingly, our study further reveals that senescence inhibition by our strategy is mainly attributed to downregulation of the splicesome, proteasome formation, and pyrimidine metabolism signaling pathways without inhibition of mitochondrial function, increased autophagy, and maintenance of telomere length. However, future studies should more clearly elucidate the molecular mechanisms by which coinhibition of activated p38α and mTORC1 prevents ex vivo expanded hUCB $CD34^+$ cells from undergoing senescence.

5 | CONCLUSION

Our study demonstrates that coinhibition of activated p38α and mTORC1 promotes stemness maintenance of HSCs from SR1-expanded hUCB $CD34^+$ cells via senescence inhibition. Thus, we established a new strategy to maintain the stemness of ex vivo differentiation inhibitor-expanded human HSCs via coinhibition of multiple independent senescence initiating signal pathways. This senescence inhibition-induced stemness maintenance of ex vivo expanded HSCs could also play an important role in other HSC expansion systems.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

X.L., X.M., Y.C.: performed the experiments, analyzed the data, and wrote the manuscript; D.P., H.W.: participated in the collection of bone marrow and spleen cells from the NOG mice; S.C.: collected hUCB from healthy human puerperas; Y.X., L.L., Y.G., H.Z., F.C., T.C.: critically evaluated the experimental data and the manuscript; J.C.: carried out the enrichment analysis of transcriptome data; L.L.: conceived the study, analyzed the data, and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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