Supplementary data

Supplementary Figures

Supplementary Figure S1. p38α and mTORC1 signaling pathways are activated during ex vivo hematopoietic growth factors (HGFs) expanded hUCB CD34+ cells. hUCB CD34+ cells were expanded ex vivo with HGFs for different days (0, 2, 4, 6, and 8 days). The expressions of p-P38 (a) and both p-P70 S6 and p-4E-BP1 (b) were detected by western blotting to examine the activation of p38α and mTORC1, respectively.

Supplementary Figure S2. Activated P38α and mTORC1 signaling pathways are inhibited by LY2228820 (LY) or rapamycin (Rapa), respectively. hUCB CD34+
cells were cultured *ex vivo* with HGFs along with 0.1% DMSO (vehicle) or LY and/or Rapa. After 7 days, uncultured cells and their expanded progeny were stained with Alexa Fluor 488 -linked antibodies against p-P38 or p-S6. The expression of p-P38 (a) and p-S6 (b) in uncultured and expanded CD34+ cells were determined by flow cytometry (n = 3 for all). ***p < 0.001, **p < 0.01.

**Supplementary Figure S3**

Supplementary Figure S3. Coinhibition of activated p38α and mTORC1 signaling pathways further inhibit senescence in *ex vivo* expanded CD34+ cells. hUCB CD34+ cells were cultured *ex vivo* for 7 days with HGFs along with 0.1% DMSO (vehicle) or LY and/or Rapa to inhibit activated p38α and/or mTORC1, respectively. (a) SA-β-gal activity in uncultured CD34+ cells and their expanded progeny was determined using C12FDG staining and flow cytometry (n=3). (b) hUCB CD34+ cells were stained with 10 μM CFSE prior to *ex vivo* expansion. The fluorescence intensity of CFSE was detected by flow cytometry after expansion (n =
4). (c) Cell cycle distribution of hUCB CD34+ cells was determined using PI staining and flow cytometry (n = 4). (d) Apoptosis of hUCB CD34+ cells was analyzed by Annexin V and PI staining and flow cytometry (n = 4). *** p < 0.001, ** p < 0.01 and * p < 0.05; NS, no significance.

**Supplementary Figure S4**

Supplementary Figure S4. Coinhibition of activated p38α and mTORC1 further promotes maintenance but does not increase expansion of hUCB-derived phenotypic HSCs than inhibition of p38α or mTORC1, respectively. hUCB CD34+ cells were cultured ex vivo for 7 days with HGFs along with 0.1% DMSO (vehicle) or LY and/or Rapa to inhibit activated p38α and/or mTORC1, respectively. (a) Representative FACS plots of the CD34+CD38−, CD34+CD90+ and CD34+CD45RA− subpopulations after expansion are shown. (b) Representative FACS
plot of the CD34⁺CD38⁻CD90⁻CD45RA⁻ subpopulation after expansion is shown. The proportions (e-f) or fold expansions (g-j) of CD34⁺CD38⁻, CD34⁺CD90⁺, CD34⁺CD45RA⁻ and CD34⁺CD38⁻CD90⁻CD45RA⁻ cells in the progeny of CD34⁺ cells expanded ex vivo for 7 days were determined by flow cytometry or calculated, respectively (n = 3). Fold expansion of subpopulations was calculated following this method: fold expansion = total cells × percentage of subpopulations after culture / input cells (3*10⁴) × percentage of subpopulations before culture. *** p < 0.001, ** p < 0.01 and * p < 0.05; NS, no significance.

Supplementary Figure S5

Supplementary Figure S5. Coinhibition of activated p38α and mTORC1 does not promote HPCs expansion. The progeny of expanded hUCB CD34⁺ cells following the above mentioned method were diluted and cultured in MethoCult™ H4435 enriched medium for 14 days to form CFUs. Representative images (a) and statistical plots (b) of CFU-E, CFU-GM, CFU-M, CFU-GEMM and total CFUs are shown (n =
6). *** p < 0.001, ** p < 0.01, and * p < 0.05; NS, no significance.

**Supplementary Figure S6**

Supplementary Figure S6. Coinhibition of activated p38α and mTORC1 signaling pathways promotes maintenance of phenotypic HSPC in SR1-expanded hUCB CD34+ cells in vitro. hUCB CD34+ cells were cultured ex vivo for 7 days with
HGFs along with 0.1% DMSO (vehicle) or SR1 and/or inhibitors of p38α (LY) and mTORC1 (Rapa). The cultured cells were then collected for phenotypic analysis. Representative FACS plots for the CD34+ (a), and CD34−CD38−, CD34+CD90+, CD34−CD45RA− and CD34−CD38−CD90−CD45RA− subpopulations (b) are shown.

**Supplementary Figure S7**

Supplementary Figure S7. **Coinhibition of activated p38α and mTORC1 does not increase the total number of SR1-expanded cells.** hUCB CD34+ cells were cultured *ex vivo* for 7 days with HGFs along with 0.1% DMSO (vehicle) or SR1 and/or inhibitors of p38α (LY) and mTORC1 (Rapa). The fold expansion of all cells was determined after 7 days of culture by cell counting using 0.4% trypan blue under an inverted microscope (n = 5). ***p < 0.001; NS, no significance.
Supplementary Figure S8. Coinhibition of activated p38α and mTORC1 increases the engraftment of ex vivo SR1-expanded hUCB CD34+ cells in the blood of NOG mice. A total of 1000, 3000, 10000, and 30000 uncultured hUCB CD34+ cells or a fraction of the final culture equivalent to 1000, 3000, and 10000 starting hUCB CD34+ cells were transplanted into NOG mice. At weeks 1, 4, 8, peripheral blood was collected from retroorbital sinuses and used to detect the engraftment of donor cells in the blood of NOG mice. Representative FACS plot (a) of engraftment at week 8 in the blood of NOG mice of 10000 non-cultured hUCB CD34+ cells or a fraction of the final culture equivalent to 10000 starting hUCB CD34+ cells cultured under different conditions. Statistical plots (b) of engraftment at week 8 in the blood of NOG mice of 1000~30000 uncultured hUCB CD34+ cells or a fraction of the final culture equivalent to 1000~10000 starting hUCB CD34+ cells cultured under different conditions. Each data point represents an independent mouse,
and the short line for each data set represents the mean engraftment.

Supplementary Figure S9

Supplementary Figure S9. Progeny of hUCB CD34⁺ cells expanded in the presence of SR1 and inhibitors of both activated p38α and mTORC1 possess the ability to engraft in the spleens of NOG mice. A total of 1000, 3000, 10000, and 30000 uncultured hUCB CD34⁺ cells or a fraction of the final culture equivalent to 1000, 3000, and 10000 starting hUCB CD34⁺ cells were transplanted into NOG mice for 13 weeks. The mice were sacrificed, and their spleens were isolated and used to determine engraftment of hUCB CD34⁺ cells. (a) Representative FACS plot and (b) the percentage of engrafted human B cells (hCD19⁺mCD3⁻) and (c) the percentage of engrafted human donor cells (hCD45⁺mCD45⁻) in the spleen of NOG mice transplanted with 10000 uncultured hUCB CD34⁺ cells or a fraction of the final culture equivalent to 10000 starting hUCB CD34⁺ cells are shown. Each data point represents an independent mouse, and the short line for each data set represents the
Human red blood cell progenitors, megakaryocytes and NK cells are reconstituted in the bone marrow of NOG mice transplanted with the progeny of hUCB CD34+ cells expanded ex vivo in the presence of SR1 and inhibitors of both activated p38α and mTORC1. A total of 1000, 3000, 10000, and 30000 non-cultured hUCB CD34+ cells or a fraction of the final culture equivalent to 1000, 3000, and 10000 starting hUCB CD34+ cells were transplanted into NOG mice for 13 weeks. The mice were sacrificed, and their bone marrow was isolated and used to detect engraftment. The engraftment of human red blood cell progenitors (a), megakaryocytes (b) and NK cells (c) in bone marrow of NOG mice transplanted with 10000 non-cultured hUCB CD34+ cells or a fraction of the final culture equivalent to 10000 starting hUCB CD34+ cells are shown. Each data point represents an independent mouse, and the short line for each data set represents the mean engraftment.
Supplementary Figure S11. Coinhibition of activated p38α and mTORC1 along with SR1 treatment promotes the multiple lineages hematopoietic reconstitution and phenotypic HSC maintenance abilities of the progeny of *ex vivo* expanded hUCB CD34+ cells in the bone marrow of NOG mice at week 13. A total of 1000, 3000, 10000, and 30000 uncultured hUCB CD34+ cells or a fraction of the final culture equivalent to 1000, 3000, and 10000 starting hUCB CD34+ cells were transplanted into NOG mice for 13 weeks. Then the mice were sacrificed, and their bone marrow were isolated and used to analyze engraftment. Representative FACS plots of human donor cells (hCD45+ mCD45-) (a), human T (hCD3+hCD19+) and B (hCD3+hCD19+) lymphoid cells (b), myeloid cells (hCD33+hCD11b+) (c), NK cells (hCD3+hCD56+) (d), red blood cell progenitors (hGly-A+) and megakaryocytes (hCD41+) (e), and phenotypic HSCs (CD34+CD38-, CD34+CD90+ and CD34+CD45RA- subpopulations) (f-h) reconstituted by the engraftment of 10000 uncultured hUCB CD34+ cells or a fraction of the final culture equivalent to 10000 starting hUCB CD34+ cells in bone marrow of NOG mice are shown.
Supplementary Figure S12. Coinhibition of activated p38α and mTORC1 increases the number of SRC in 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 were transplanted into NOG mice for 16 weeks. Then mice were sacrificed, and half of the bone marrow cells from primary recipient mice were transplanted into secondary recipient NOG mice for another 16 weeks. The chimerism of human donor cells (hCD45+mCD45−) in the bone marrow of the primary (a) and secondary (b) recipient NOG mice are shown. Each data point represents an independent mouse, and the short
line for each data set represents the mean engraftment.

**Supplementary Tables**

**Supplementary Table S1. Summary of primary and secondary NOG engraftment**

| Groups  | transplanted cell dose (or equivalent starting dose for cultured cells) | reconstituted mice / primary recipients | reconstituted mice / secondary recipients |
|---------|-------------------------------------------------------------------------|----------------------------------------|------------------------------------------|
|         | 1000                                                                    | 4/8                                    | 1/4                                      |
|         | 3000                                                                    | 6/8                                    | 2/6                                      |
|         | 5000                                                                    | 7/8                                    | 3/5                                      |
| uncultured | 1000                                                                    | 2/8                                    | 1/8                                      |
|         | 3000                                                                    | 4/5                                    | 2/4                                      |
| vehicle | 5000                                                                    | 6/8                                    | 3/6                                      |
|         | 1000                                                                    | 4/8                                    | 1/4                                      |
|         | 3000                                                                    | 3/4                                    | 1/3                                      |
| LY-Rapa | 5000                                                                    | 4/4                                    | 3/3                                      |
|         | 1000                                                                    | 4/7                                    | 0/4                                      |
| SR1     | 3000                                                                    | 5/7                                    | 3/5                                      |
|         | 1000                                                                    | 6/7                                    | 3/6                                      |
|         | 3000                                                                    | 6/6                                    | 3/3                                      |
| LY-Rapa+SR1 | 5000                                                                    | 7/7                                    | 3/3                                      |

**Supplementary Table S1. Summary of primary and secondary NOG engraftment.**

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 were transplanted into sublethally irradiated (230 cGy) NOG mice via the tail vein within 24 h 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. Reconstitute mice was determined by the
presence of greater than 0.1% of hCD45+ mCD45- cells in recipient bone marrow.

**Supplementary Table S2. Poisson statistics for primary engraftment**

| Groups       | SRC frequency per CD34+ starting cells | 95% confidence interval of SRC frequency | p-value | SRC content per 1×10^6 CD34+ starting cells | 95% confidence interval of SRC content |
|--------------|----------------------------------------|------------------------------------------|---------|-------------------------------------------|---------------------------------------|
| uncultured   | 1/2040                                  | 1/1178-1/3534                            | ≤0.01   | 49                                        | 28-84                                 |
| vehicle      | 1/2998                                  | 1/1629-1/5515                            | ≤0.01   | 33                                        | 18-61                                 |
| LY+Rapa      | 1/1580                                  | 1/788-1/3169                            | ≤0.05   | 63                                        | 31-126                                |
| SR1          | 1/1707                                  | 1/888-1/3285                            | ≤0.05   | 58                                        | 30-112                                |
| LY+Rapa+SR1  | 1/503                                  | 1/209-1/1211                            | —       | 198                                       | 82-478                                |

**Supplementary Table S2. Poisson statistics for primary engraftment.** Poisson statistics was applied to the data in supplementary table S1. SRC frequency and statistical differences between various groups were determined using L-Calc software. The number of SRC in 10^5 hUCB-derived CD34+ cells is calculated.
### Supplementary Table S3. Poisson statistics for secondary engraftment

| Groups   | SRC frequency per CD34⁺ starting cells | 95% confidence interval of SRC frequency | p-value | SRC content per $1 \times 10^5$ CD34⁺ starting cells | 95% confidence interval of SRC content |
|----------|----------------------------------------|-----------------------------------------|---------|---------------------------------------------------|----------------------------------------|
| uncultured | 1/5776 | 1/2548-1/13097 | $p<0.01$ | 17 | 7-39 |
| vehicle   | 1/6294 | 1/2786-1/14222 | $p<0.01$ | 15 | 7-35 |
| LY+Rapa   | 1/3283 | 1/1324-1/8140 | $p<0.05$ | 30 | 12-75 |
| SR1       | 1/3244 | 1/1438-1/7317 | $p<0.05$ | 30 | 13-69 |
| LY+Rapa+SR1 | 1/1136 | 1/497-1/2596 | — | 88 | 38-201 |

**Supplementary Table S3. Poisson statistics for secondary engraftment.** Poisson statistics was applied to the data in supplementary table S1. SRC frequency and statistical differences between various groups were determined using L-Calc software. The number of SRC in $10^5$ hUCB-derived CD34⁺ cells is calculated.