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Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal

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Abstract

The small number of hematopoietic stem and progenitor cells in cord blood units limits their widespread use in human transplant protocols. We identified a family of chemically related small molecules that stimulates the expansion ex vivo of human cord blood cells capable of reconstituting human hematopoiesis for at least 6 months in immunocompromised mice. The

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SUPPLEMENTARY MATERIALS

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We report no conflict of interest.

potent activity of these newly identified compounds, UM171 being the prototype, is independent of suppression of the aryl hydrocarbon receptor, which targets cells with more-limited regenerative potential. The properties of UM171 make it a potential candidate for hematopoietic stem cell transplantation and gene therapy.

Allogeneic HSC transplant is the only curative therapy for numerous hematologic malignancies. Unfortunately 30 to 40% of patients will not have a human leukocyte antigen (HLA)–identical donor and will be excluded from therapy (1). Cord blood (CB) transplants offer several advantages, namely, the reduced need for HLA matching [thereby extending transplantation availability to nearly all patients (2)] and the decreased risk of chronic graft-versus-host disease, the most important determinant of long-term quality of life in transplant patients. However, CB transplants suffer from limited progenitor cell dose, leading to delayed neutrophil engraftment and increased mortality (3, 4).

Recent studies in immunodeficient mice have confirmed the existence of human CB-derived long-term-repopulating hematopoietic stem cells (LT-HSCs) capable of regenerating the lifelong production of all mature blood cells (5). These LT-HSCs show a delayed engraftment pattern, in opposition to short-term HSCs (ST-HSCs) that produce short-lived progenitors responsible for the production of mature blood cells and prompt neutrophil recovery (3, 5). Hence, there is great interest in the development of conditions for robustly expanding these progenitor cells while maintaining or expanding LT-HSCs. Unfortunately, most expansion systems available to date achieve progenitor cell expansion at the expense of the LT-HSC loss (6), increasing the risk of late graft failure.

Recent studies showed that aryl hydrocarbon receptor (AhR) antagonists and a notch ligand agonist promote the in vitro expansion of human CB cells, with repopulating activity lasting up to 16 weeks in immunodeficient mice (7, 8). We developed an automated and continuous medium delivery system that produces an equivalent expansion of CB cells with similar repopulation properties (9). This fed-batch culture system optimizes the balance of stimulatory and inhibitory factors in a small culture volume. We hypothesized that small molecules with potent LT-HSC–stimulating activities might be identified and potentiated in this fed-batch culture system.

We screened a library of 5280 low-molecular-weight compounds for their ability to expand human $CD34^+CD45RA^-$ mobilized peripheral blood (mPB) cells, which are enriched in LT-HSCs (10) (fig. S1, A and B). Seven hits were identified after excluding the autofluorescent compounds (Fig. 1A and fig. S1C), five of which were known [four (11, 12)] or previously unknown (one, UM125454, fig. S2) suppressors of the AhR pathway (Fig. 1B). The other two compounds, UM729 (fig. S2) and UM118428, did not suppress the AhR pathway (Fig. 1B). Because of its apparent superior activity in expanding $CD34^+CD45RA^-$ cells, UM729 was selected for further characterization and optimization by structure activity relationship (SAR) studies that determine the link between the chemical structure of the compound and its biological activity in expanding $CD34^+CD45RA^-$ cells. More than 300 newly synthesized analogs of UM729 were examined, of which one (UM171, Fig. 1C) was 10 to 20 times more potent than UM729, with effective concentrations of 17 to 19 nM when tested for its ability to stimulate the expansion of a HSC-enriched population, $CD34^+CD45RA^-$

cells (10) (Fig. 1D and fig. S3, A and B). UM729 did not expand mouse HSCs (fig. S4). UM729 and UM171 treatment enhanced the engraftment potential of $CD34^+$ macaque cells by threefold when compared with controls (fig. S5).

Optimization of fed-batch culture duration indicated that the highest expansion of multipotent progenitors and long-term culture-initiating cells (LTC-ICs) was obtained on day 12 (fig. S3, C to E). Likewise, the proportion of apoptotic cells was lower at that time when compared with day 16 (fig. S3F). We also observed that the effect of UM171 requires its constant presence in the media and that the molecule lacks direct mitogenic activity (fig. S6). Cell division tracking further showed that UM171 does not affect the division rate of phenotypically primitive populations (fig. S7).

We next designed experiments to compare the impacts of UM171 and SR1 on outputs of $CD34^+$ CB cells introduced in fed-batch cultures. Control (dimethyl sulfoxide, DMSO) fed-batch cultures contained mostly differentiated cells (Fig. 2A, DMSO) and a reduced frequency of $CD34^+CD45RA^-$ cells (compare red box of the two top right graphs in Fig. 2B). In contrast, this phenotype remained prominent in cultures containing UM171 (Fig. 2A and red box in Fig. 2B). Although $CD34^+$ cell frequencies in cultures containing SR1 or UM171 were similar (Fig. 2B, middle graphs), $CD34^+CD45RA^-$ cells were proportionally more abundant when UM171 was present (Fig. 2B, right-hand graph, red box; $P < 0.005$, Mann-Whitney test). Determining the absolute numbers of these primitive phenotypes and functionally defined cells confirmed the greater effect of UM171 when compared with control or SR1 [compare UM171 (red) with fed-batch (black) and SR1 (blue) in Fig. 2C and figs. S8 and S9 for fold expansion and absolute cell numbers, respectively]. Furthermore, the effect of UM171 on colony-forming unit of granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM) expansion (Fig. 2D) and on mature cell output suppression (e.g., $CD34^-$ cells in Fig. 2, B and 2C) was enhanced by the addition of SR1. Together, these observations show that these two compounds cooperate to enhance ex vivo expansion of progenitor cells and that they suppress mature cell output (differentiation). These data also suggest that UM171 targets phenotypically more primitive cells than those targeted by SR1.

By using conditions described in fig. S10A, we next determined the frequencies (adjusted to numbers of $CD34^+$ cells at day 0, hereafter called d0 equivalent) and the absolute numbers of LT-HSCs in fed-batch cultures supplemented with DMSO (control), UM171, SR1, or the combination of both (Fig. 3A and table S1, respectively). When analyzed at 20 weeks posttransplantation, LT-HSC frequencies in fresh (uncultured) $CD34^+$ CB were measured at the expected frequency of ~1 per 880 $CD34^+$ starting cells [95% confidence interval (CI) of 470 to 1600; see Fig. 3A in which frequencies (red line) and 95% CIs (gray box) are indicated]. Similar LT-HSC frequencies were obtained from fed-batch cultures (DMSO) whether or not they contained SR1 (Fig. 3A). Frequencies of d0 equivalent LT-HSCs were 13-fold higher in cultures supplemented with UM171 when compared with DMSO or to fresh (uncultured) controls (Fig. 3A). Absolute LT-HSC values determined after 20 weeks posttransplantation in all culture conditions are provided in table S1. Simultaneous addition of SR1 to UM171-treated cultures did not significantly change these numbers, indicating that the cooperativity between these two molecules is restricted to short-lived progenitors

and that LT-HSC output is selectively enhanced in the presence of UM171 (Fig. 3A and table S1).

We analyzed the nature of human hematopoietic reconstitution obtained by transplanting fresh or expanded cells in NOD *scid* gamma (NSG) mice. Levels of human cell engraftment, whether total (CD45), myeloid (CD33), or B lymphoid (CD19), were determined for ~300 mice and represented in the form of a heat map in Fig. 3B (raw data in table S2). Analysis of this data set indicates two emerging patterns of human reconstitution, one from predominantly lymphomyeloid LT-HSCs, observed at high cell doses with most conditions, and the other from LT-HSCs that display a lymphoid-deficient differentiation phenotype mostly observed with UM171 treatment, with or without SR1 (Fig. 3B). However, neither B lymphopoiesis nor the frequency or number of lymphomyeloid LT-HSCs is negatively affected by UM171 (Fig. 3B). SR1 treatment appeared to compromise the in vivo proliferative potential, although not the number, of lymphomyeloid LT-HSCs (compare reconstitution levels of SR1 with uncultured or UM171 conditions in Fig. 3B). In support of this, the presence of SR1 in UM171 treated cultures appears to slightly hamper the proliferative potential of the expanded cells (see reduction in red in Combi versus UM171 conditions in Fig. 3B). The impact of UM171 on LT-HSC was preserved at 30 weeks posttransplantation (fig. S10B and table S3), at which time multilineage contribution remained obvious at the high cell dose (Fig. 3C). At this extended time point post-transplant, we also noted a slight augmentation in myeloid cell output, a phenomenon recently described with normal unexpanded cells (5, 13). The molecular and cellular mechanisms underlying this effect of UM171 on expanding LT-HSCs that show a lymphoid-deficient differentiation pattern are of interest given previous studies of a similar self-perpetuating LT-HSC subset in mice (14) whose prominence is increased in the bone marrow as soon as HSCs begin to migrate from the fetal liver to that site (15).

To further evaluate the impact of UM171-treated LT-HSC population(s), we performed transplantation experiments in secondary recipients. For these studies, four to six primary recipients were selected per condition in which human reconstitution ranged between 10 and 70%. Results (table S4) indicate that UM171 ex vivo treatment did not appear to affect the capability of LT-HSC to expand in primary recipients and hence similarly reconstituted secondary animals for at least 18 more weeks, thus indicating that cells exposed to the molecule ex vivo are still competent in secondary recipients, where they show no advantage when compared to unmanipulated $CD34^+$cells.

We next performed RNA sequencing (RNA-seq) expression profiling experiments to gain insights into the mode of action of UM171. SR1-treated cells were also analyzed for comparison. As expected, SR1 but not UM171 treatment resulted in down-regulation of AhR target genes such as *CYP1B1*, *CYP1A1*, and *AhRR* (Fig. 4A and fig. S11A) (7, 16). Unlike SR1, UM171 treatment was accompanied by a marked suppression of transcripts associated with erythroid and megakaryocytic differentiation (Fig. 4B and fig. S11B). Only six to seven genes were commonly up- or down-regulated in cells exposed to UM171 or SR1 (fig. S12A). In line with these results, gene expression signatures were very different between cells exposed to UM171 versus those treated with SR1 (fig. S11C and fig. S12B). Most notably, we found that the transmembrane protein of unknown function, TMEM183A,

was the most up-regulated transcript in both conditions (fig. S11, A and B) and that the most highly up-regulated genes in UM171-treated cells encode for surface molecules (fig. S11B, highlighted in red). These genes include *PROCR* (also called EPCR or CD201), which represents a known marker of mouse LT-HSCs (17). Additional RNA-seq experiments and fluorescence-activated cell-sorting (FACS) analyses confirmed that expression of this receptor is modulated, in a dose-dependent manner, by UM171 treatment (fig. S13).

UM171 enables a robust ex vivo expansion of human CB cells with functionally validated long-term in vivo repopulating capability (Fig. 4C). On the basis of these findings, we suggest that UM171 acts by enhancing the human LT-HSC self-renewal machinery independently of AhR suppression. Conversely, AhR inhibitors' activity appears restricted to the production of cells with less-durable self-renewal activity (Fig. 4C). By expanding LT-HSCs and downstream cells in vitro using UM171, it may become possible for small, well HLA-matched CB units to become a prioritized source of cells for transplantation in future donor selection algorithms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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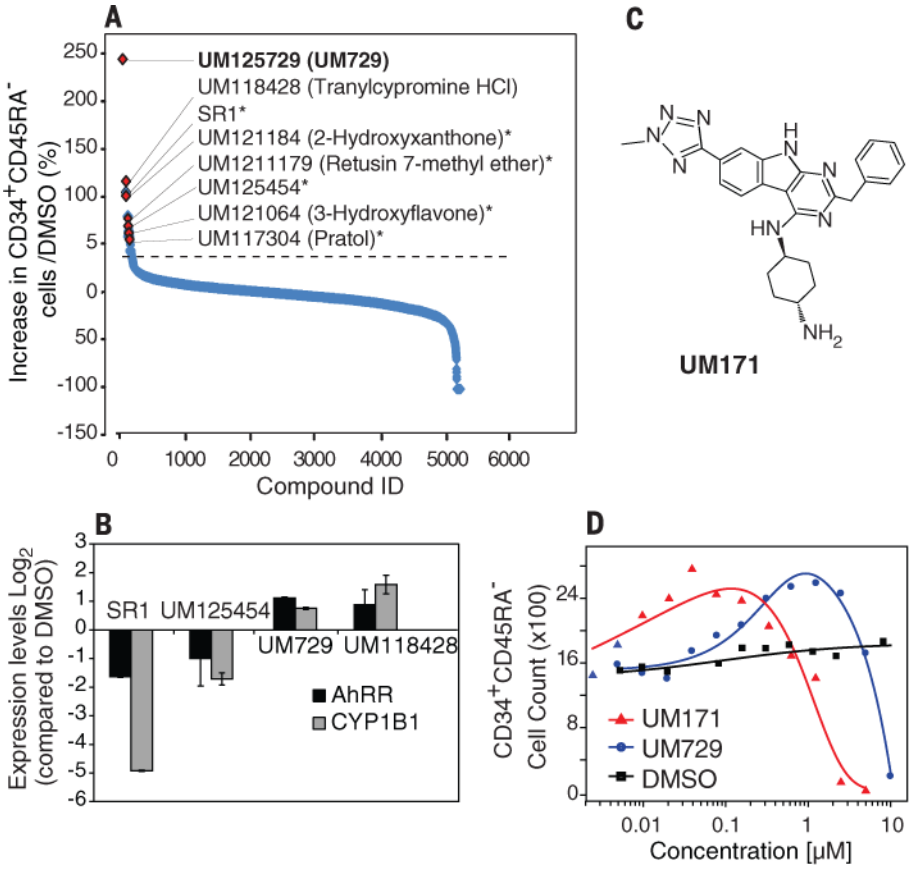

Fig. 1. Identification of previously unknown compounds promoting human CD34$^+$ cell expansion

(**A**) Results of primary screen; asterisks denote the compounds that suppress the AhR pathway. (**B**) Changes in expression levels of AhR targets (AhRR and CYP1B1) measured by quantitative reverse transcription polymerase chain reaction after a 12-hour incubation with selected compounds compared with DMSO (using glyceraldehyde-3-phosphate dehydrogenase and hypoxanthine-guanine phosphoribosyltransferase as control, mean T SD). (**C** and **D**) Chemical structure of UM171, the optimized version of UM729, and their comparative activity on expansion of CD34$^+$CD45RA$^-$ mPB cells after 7-day cultures. The cytostatic/cytotoxic effects of UM729 and UM171 were observed at values above 1 and 0.125 μM, respectively.

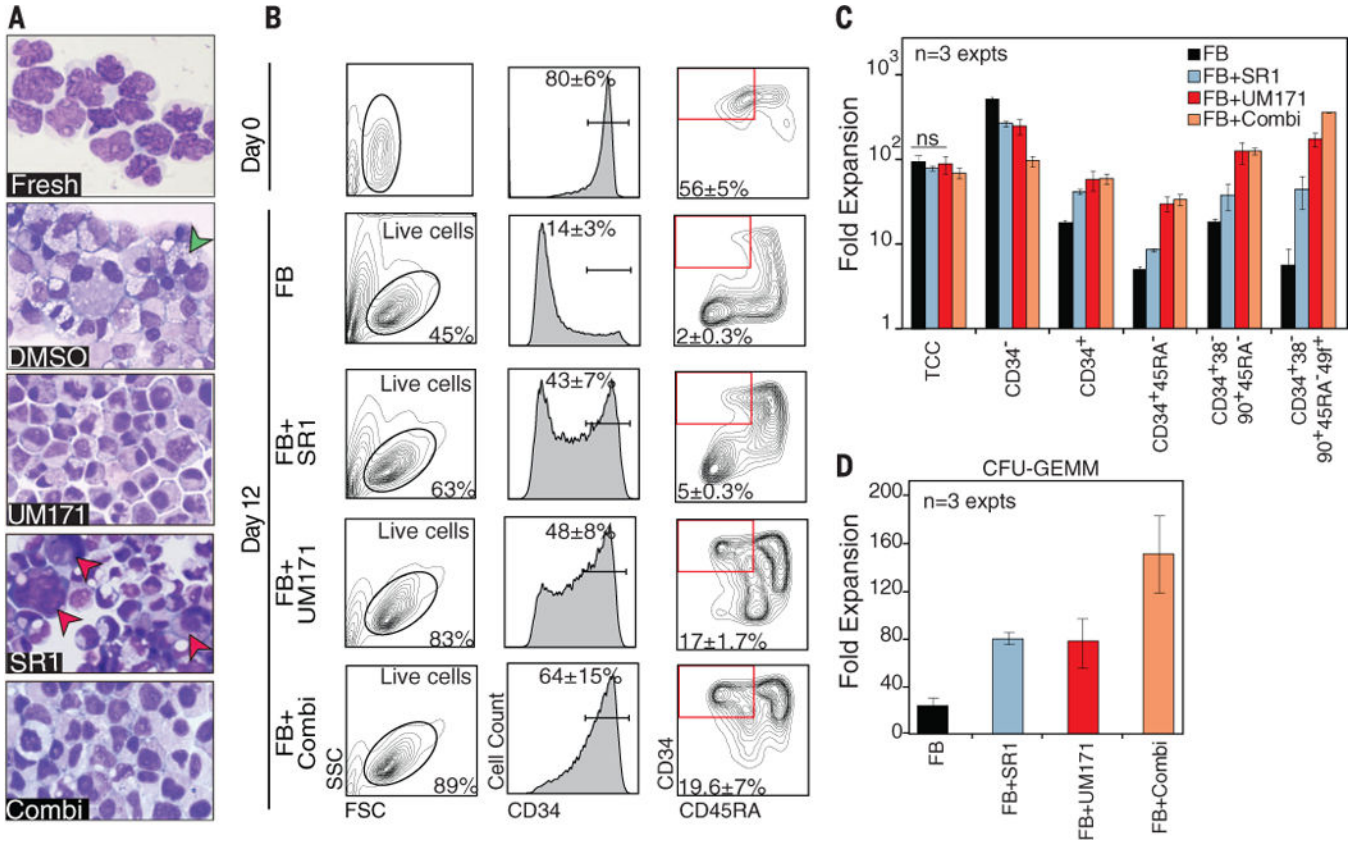

Fig. 2. UM171 attenuates cell differentiation and promotes ex vivo expansion of primitive human hematopoietic cells

(**A**) Wright-stained cytospin preparation of $CD34^+$ CB cells at day 0 and after 12 days in fed-batch cultures supplemented with vehicle (DMSO 0.1%), UM171 (35 nM), SR1 (750 nM), or a combination of SR1 (500 nM) and UM171 (35 nM). Arrowheads show macrophages (green) and megakaryocyte (red). (**B**) Representative FACS profiles of $CD34^+$ and $CD34^+CD45RA^-$ populations in fresh (day 0) or cultured (day 12) CB cells. (**C**) The fold expansion of phenotypically defined cell subsets after 12 days in fed-batch cultures supplemented with indicated compounds [mean ± SD unless specified (ns, not significant); all values are significant when compared with control (black bars): $P < 0.05$, Mann-Whitney test]. (**D**) Fold expansion of CFU-GEMMs after 12 days in cultures.

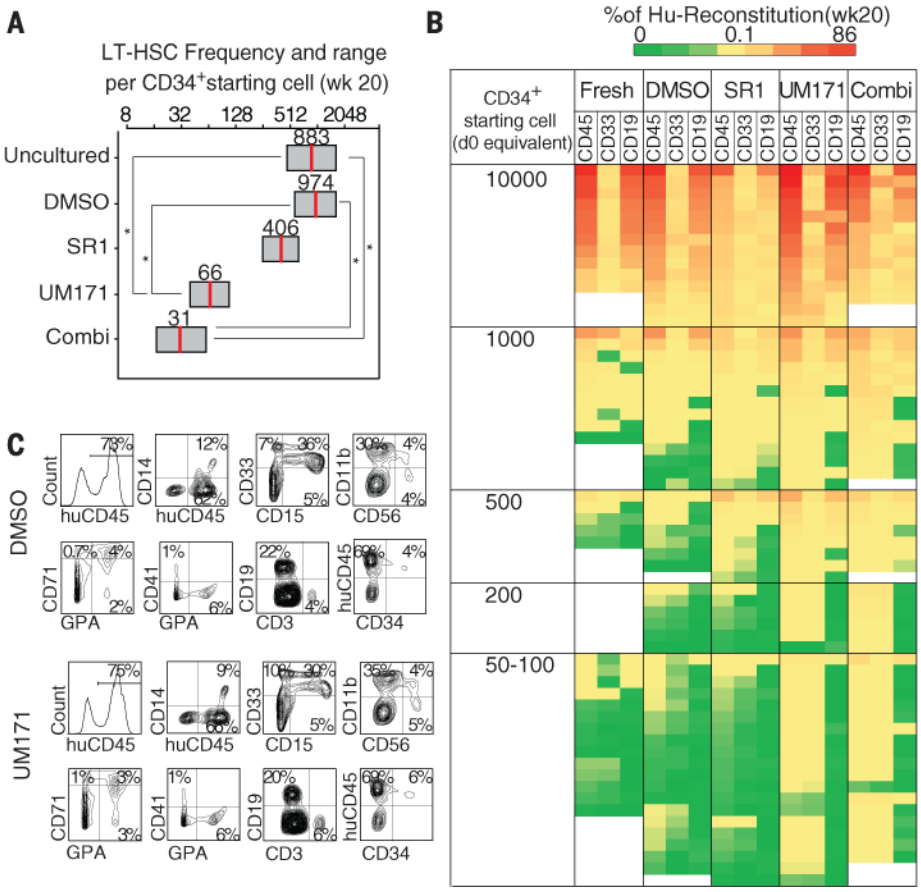

Fig. 3. UM171 promotes expansion of LT-HSCs

(**A**) LT-HSC frequencies (red lines) and 95% CIs (gray boxes) presented as 1/number of starting cell (day 0) equivalent for each condition; $n = 5$ independent experiments performed with a pool of two to three human CB units per experiment. Significance level $*P < 0.05$ (Mann-Whitney test). (**B**) Levels of human (Hu) engraftment in NSG mice transplanted with different cell doses (column 1); red, 86%; green, 0%. See table S2 for raw data. (**C**) Representative FACS profiles showing multilineage repopulation of NSG mice (GPA, glycophorin A or CD235a).

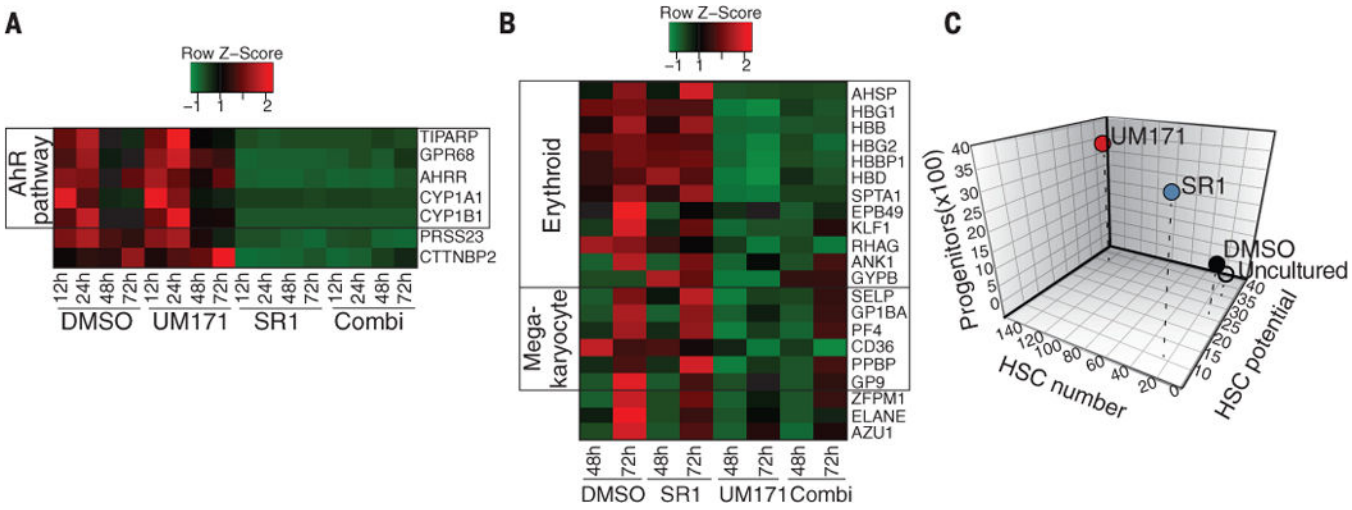

Fig. 4. Summary of UM171 effect on cell expansion and differentiation

(**A**) Heat map showing expression of AhR targets (A) or lineage-specific (**B**) genes (green, low; red, high) in indicated conditions. (**C**) Comparison of UM171 with SR1 and DMSO on number and quality of LT-HSCs and progenitors based on results from expansion of 10,000 fresh $CD34^+$ CB cells.