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CAPE promotes the expansion of human umbilical cord blood-derived hematopoietic stem and progenitor cells *in vitro*

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Due to the low number of collectable stem cells from single umbilical cord blood (UCB) unit, their initial uses were limited to pediatric therapies. Clinical applications of UCB hematopoietic stem and progenitor cells (HSPCs) would become feasible if there were a culture method that can effectively expand HSPCs while maintaining their self-renewal capacity. In recent years, numerous attempts have been made to expand human UCB HSPCs *in vitro*. In this study, we report that caffeic acid phenethyl ester (CAPE), a small molecule from honeybee extract, can promote *in vitro* expansion of HSPCs. Treatment with CAPE increased the percentage of HSPCs in cultured mononuclear cells. Importantly, culture of $CD34^+$ HSPCs with CAPE resulted in a significant increase in total colony-forming units and high proliferative potential colony-forming units. Burst-forming unit-erythroid was the mostly affected colony type, which increased more than 3.7-fold in 1 μg mL^{-1} CAPE treatment group when compared to the controls. CAPE appears to induce HSPC expansion by upregulating the expression of SCF and HIF1-α. Our data suggest that CAPE may become a potent medium supplement for *in vitro* HSPC expansion.

hematopoietic stem and progenitor cells, caffeic acid phenethyl ester, expansion

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Transplantation of hematopoietic stem cells and progenitor cells (HSPCs) has been used for many years to treat various malignant and non-malignant hematologic diseases. The infused HSPCs can replace and reconstitute the hematopoietic and immune systems in patients subjected to chemotherapy or radiotherapy. In the last decade, umbilical cord blood (UCB) becomes a new resource of HSPCs for allogeneic transplantation [1]. UCB transplantation shows many advantages, including elimination of donor risk, rapid availability, low risk of infection, and preservation of graft-versus-leukemia effect, despite the relatively low risk of graft-versus-host disease. Due to the low number of collectable stem cells from single UCB units, the initial uses were limited to pediatric therapies. Clinical applications of UCB HSPCs would become feasible if there were a culture method that can effectively expand HSPCs while maintaining their self-renewal capacity.

In recent years, numerous attempts have been made to expand human UCB HSPCs *in vitro* [2,3]. Most *ex vivo* cultures of human HSPCs use cytokine mixtures in serum-free medium that includes SCF, TPO, IL-3, IL-6 and Flt3-L [4]. The manipulation of some signaling pathways such as Notch and Wnt has also shown effectiveness for *ex vivo* expansion of HSPCs [5]. Several labs explored to expand HSPCs with aryl hydrocarbon receptors, copper chelators, stromal support, and automated continuous perfusion of culture systems or “bioreactors” [3,6–8]. Over-expression of transcription factors, such as SALL4, HOXB4, can

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also increase the number of HSPCs *in vitro* [9,10]. Although *in vitro* culture method for expansion of HSPCs has been improved, it still needs to be optimized to obtain more transplantable HSPCs.

Caffeic acid phenethyl ester (CAPE) is an active component of propolis from honeybee with various beneficial properties, such as anti-inflammatory and antioxidant effects [11]. Increasing evidence suggests that CAPE can exert protective effect against ischemia/reperfusion (I/R) injury occurred in many tissues [12–14]. Wang has reported that CAPE treatment induced the upregulation of kit ligand (SCF) and heme oxygenase-1 (HO-1) gene expression in HUVECs [15]. Both SCF and HO-1 play important roles in the regulation of HSPC function [16]. Thus, we speculate that CAPE may also play a role in the *in vitro* proliferation of HSPCs.

In this study, we report that CAPE, a small molecule from honeybee extract, can promote *in vitro* UCB HSPC expansion, and CAPE induces HSPC expansion probably through upregulating the expression of SCF and HIF1-α. Our data suggest that CAPE may become a potent medium supplement for *in vitro* HSPC expansion.

1 Materials and methods

1.1 The isolation of MNCs and CD34$^+$ cells

MNCs were isolated from the fresh UCB samples using lymphocyte separation medium in 1.077 g mL^{-1} (TBD sciences, China). The cell suspension was centrifuged at 600×*g* for 25 min, and then MNCs band at the interface was isolated, and washed twice with PBS (Gibco, USA). The CD34$^+$ cells were isolated from MNCs using the CD34$^+$ Microbead kit (MiltenyiBiotec, Germany) according to the manufacturer's protocol.

1.2 *In vitro* culture

The CD34$^+$ cells (2×10^5 cells/well) or MNCs (1×10^6 cells/well) were cultured in 24-well plates (Costar tissue-culture treated polystyrene, Corning, USA) in HSPC expansion medium (StemSpan SFEM, StemCell Technologies, Canada). The medium was supplemented with several cytokines (Peprotech, USA), including SCF (25 ng mL^{-1}), TPO (10 ng mL^{-1}), IL-3 (20 ng mL^{-1}), IL-6 (10 ng mL^{-1}). CAPE was added into the culture medium at different concentrations (0.1 μg mL^{-1}, 1 μg mL^{-1}). Cells were cultured at 37°C in 5% CO_2.

1.3 Flow cytometry

Cells were washed twice in PBS containing 2% FBS. The cells were incubated with isotypic antibodies or the indicated antibodies in the dark for 40 min at 4°C. Next, the cells were washed three times with PBS and suspended in 0.4 mL PBS for analysis. Live cells were determined by 7-AAD (BD Biosciences, USA) exclusion. Flow cytometry analysis was performed using BD FACS Aria (BD Biosciences). The data were analyzed using BD FACSDiva software. The following antibodies were used: CD34-PE (eBioscience, USA), CD38-APC (eBioscience).

1.4 Cell apoptosis and survival analysis

The CD34$^+$ cells were washed in PBS and apoptosis was evaluated as the percentage of Annexin V-positive cells using apoptosis kit (Invitrogen, USA) according to the manufacturer's protocol.

1.5 Colony-forming units (CFU) assay

Cultured CD34$^+$ cells (5×10^2 cells/well) or MNCs (4×10^3 cells/well) at day 7 were plated in a methylcellulose-based medium (MethoCult H4434, StemCell Technologies). Each experiment was performed in triplicate. Two weeks after plating, CFU were counted under an inverted microscope and identified according to morphological criteria. High proliferative potential colony-forming units (HPP-CFU) were counted at three weeks after plating.

1.6 Quantitative RT-PCR

Cells were lysed and total RNA was isolated by Trizol Reagent (Invitrogen), followed by reverse transcription of 1 μg total RNA with the reverse transcriptase M-MLV (Takara, Japan) using oligo(dT) primers. Quantitative RT-PCR reactions were performed with SYBR Green real time PCR Master Mix (TOYOBO, Japan) on Bio-Rad iQ5 System (Bio-Rad, USA). The expression of each gene was normalized to GAPDH gene expression and three replicates were analyzed for each condition. The primer sequences for quantitative RT-PCR were listed in Supporting Infromation (Table S1 in Supporting Information).

1.7 Statistic analysis

All values were shown as mean±SD. The statistic significance between two groups was determined by Student's *t*-test.

2 Results

2.1 Treatment with CAPE increases the percentage of HSPCs

To evaluate whether CAPE (Figure 1A) has the capability to expand HSPCs, we isolated UCB-derived MNCs and placed them in medium with cytokines alone or cytokines plus 0.1 or 1 μg mL^{-1} CAPE for 7 d. The increase in total

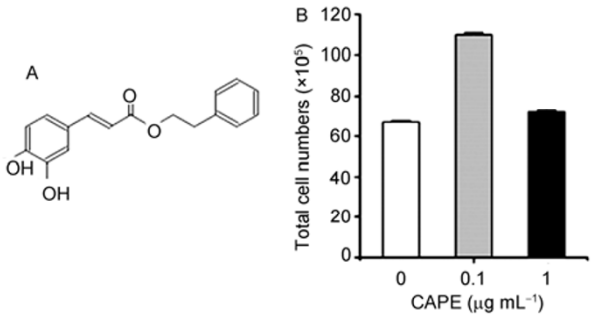

Figure 1 A, The structure of CAPE. B, The total cell numbers of MNCs growing at different CAPE concentrations for 7 d.

cell numbers was not significantly different between the cytokines alone or cytokines plus 1 μg mL^{-1} CAPE group (Figure 1B). Then we used flow cytometer to analyze the percentage of HSPCs in cultured cells. The percentage of $CD34^+$ and $CD34^+CD38^-$ cells was approximately 1.5% and 0.2% in primary UCB MNCs (Figure 2Aa). Both cytokines and cytokines plus CAPE treatments induced significant increase of the proportion of HSPCs in cultured MNCs (Figure 2Ab–d). Much higher percentage of $CD34^+$ or $CD34^+CD38^-$ cells was observed in cultures treated with cytokines plus 1 μg mL^{-1} CAPE (Figure 2Ad). The numbers of $CD34^+$ or $CD34^+CD38^-$ cells also increased in cultured

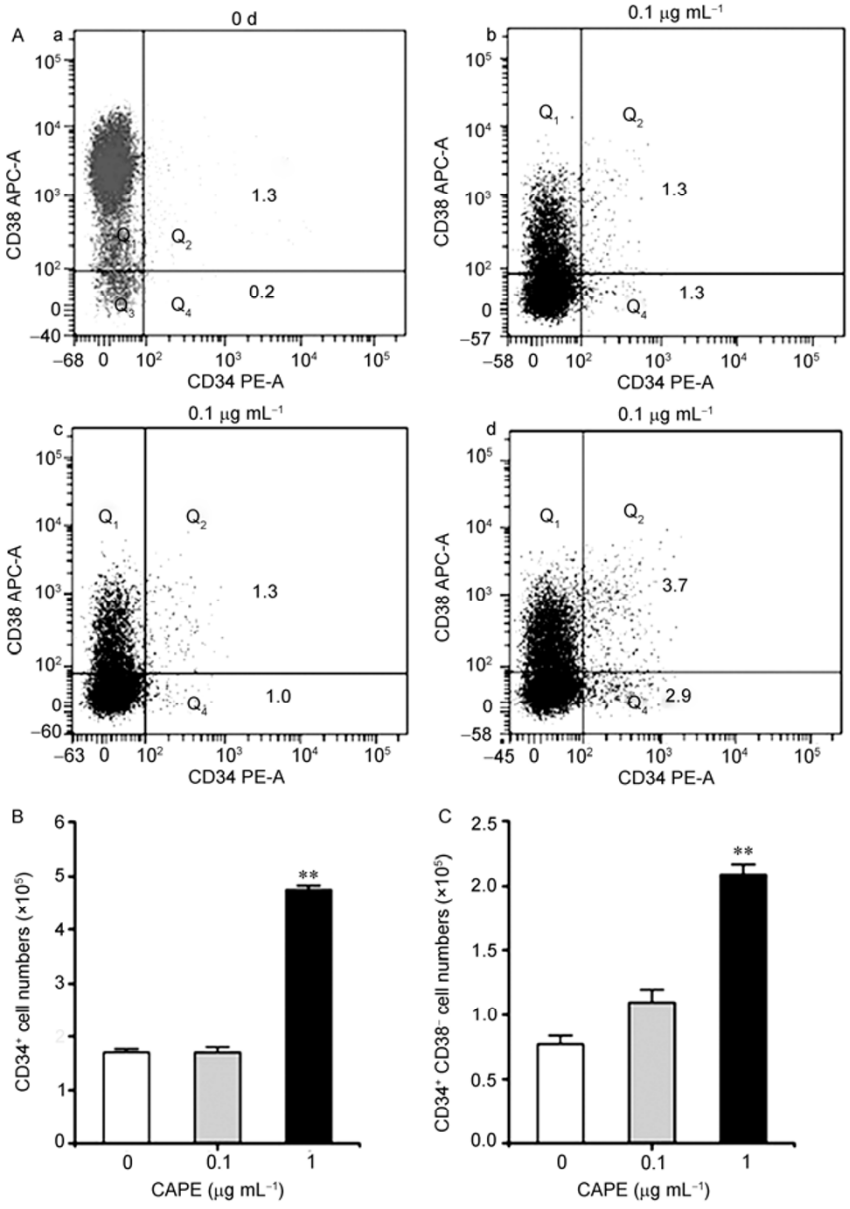

Figure 2 The detection of $CD34^+$ and $CD34^+CD38^-$ cells in cultured MNCs at different CAPE concentrations. A, The percentage of $CD34^+$ and $CD34^+CD38^-$ cells in primary MNCs (a, 0 d; just separated from cord blood) and MNCs cultured for 7 d (b–d, with different CAPE concentrations). B, The $CD34^+$ cell numbers were calculated in the cultured MNCs with CAPE treatment at different concentrations. C, The $CD34^+CD38^-$ cell numbers were calculated in the cultured MNCs with CAPE treatment at different concentrations. **, $P \leqslant 0.01$ vs. 0 μg mL^{-1}.

MNCs with cytokines plus 1μg mL^{-1} CAPE (Figure 2B and C). These data indicated that treatment with CAPE promoted the *in vitro* expansion of UCB HSPCs.

2.2 Treatment with CAPE increases the numbers of CFU

To further evaluate the effect of CAPE on HSPC proliferation, we isolated UCB $CD34^+$ cells using miniMACS selection system and cultured them with cytokines alone or cytokines plus CAPE for 7 d. Compared with control group, the survival rate of these cultured $CD34^+$ cells was not significantly altered with CAPE treatment at 0.1 μg mL^{-1} and showed a slight decrease with CAPE treatment at 1 μg mL^{-1} (Figure 3). However, the content of hematopoietic progenitor cells was significantly increased with CAPE treatment. In CFU assays (Figure 4A), culture of $CD34^+$ cells with 0.1 or 1 μg mL^{-1} CAPE resulted in a dose-dependent increase in burst-forming unit-erythroid (BFU-E) and colony forming unit-granulocyte, erythrocyte, megakaryocyte, macrophage (CFU-GEMM) (Figure 4B). Interestingly, BFU-E was the most affected colony type, increasing more than 3.7-fold in cells treated with 1 μg mL^{-1} CAPE when comparing to the control group (Figure 4B). For total CFU, a 2.6-fold or 3.1-fold increase in 0.1 or 1 μg mL^{-1} CAPE-treated cells was observed when compared with the control group (Figure 4C). The numbers of HPP-CFU were also markedly increased with CAPE treatment (Figure 4D).

2.3 CAPE upregulates the expression of SCF and HIF-1α

To investigate the mechanism by which CAPE increased the numbers of HSPC, we performed Q-PCR analysis to detect the expression of proliferation and apoptosis-related genes with cDNA from cultured MNCs. Wang has reported that CAPE treatment induced the upregulation of kit ligand (SCF) and HO-1 in HUVECs [15]. SCF plays a critical role for early phases of hematopoiesis, and facilitates HSPC proliferation and survival [16]. Our results showed that CAPE significantly upregulated the expression of SCF. The increased secretion of SCF protein was also observed in CAPE-treated cells (data not shown). HO-1 is a critical regulator of the stress response in HSPCs via controlling the level of its substrates (heme) and bioactive products [17]. The expression of HO-1 was also significantly upregulated by CAPE. Hypoxia-inducible factor-1α (HIF-1α) is required for long-term HSC maintenance and can synergize with glucocorticoids to promote BFU-E progenitor self-renewal [18,19]. We also found that the expression level of HIF-1α was increased by CAPE treatment. In addition, there was a slight increase in the expression of BCL-2 gene in the cells treated with 1 μg mL^{-1} CAPE. These results suggested that increased expression of SCF and HIF-1α promoted the expansion of HSPCs.

3 Discussion

HSPC is probably the most extensively studied somatic stem cell. It has the capability for self-renewal. It can also differentiate into all types of mature blood cells. Methods to expand HSPCs are always accompanied with their differentiation. However, it is still difficult to obtain sufficient numbers of HSPCs for transplantation. For a broader clinical application, additional studies are necessary for a deeper understanding of the cell biology of HSPCs, the microenvironment that nurtures these cells, and finding small molecules that stimulate HSPC expansion.

In this study, we found that a small molecule, CAPE, plays a regulatory role in HSPC *in vitro* proliferation. CAPE, a honeybee extract, exerts protective effect in I/R injury. It can minimize radiation-induced hearing damage in rats [20]. Moreover, CAPE has been found to be a protective agent against chemotherapy-induced and radiotherapy-induced toxicity [21]. Further study suggested that CAPE can induce the expression of HIF-1α protein and concomitantly trans-

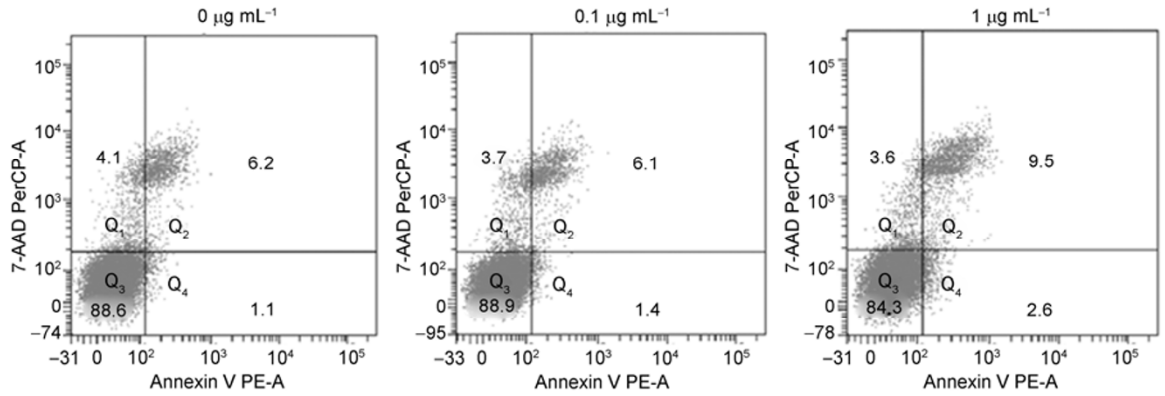

Figure 3 Survival rate analysis of MNCs growing for 7 d at different CAPE concentrations. The MNCs were stained with 7-AAD and Annexin V. Viable cells are in the lower left quadrant.

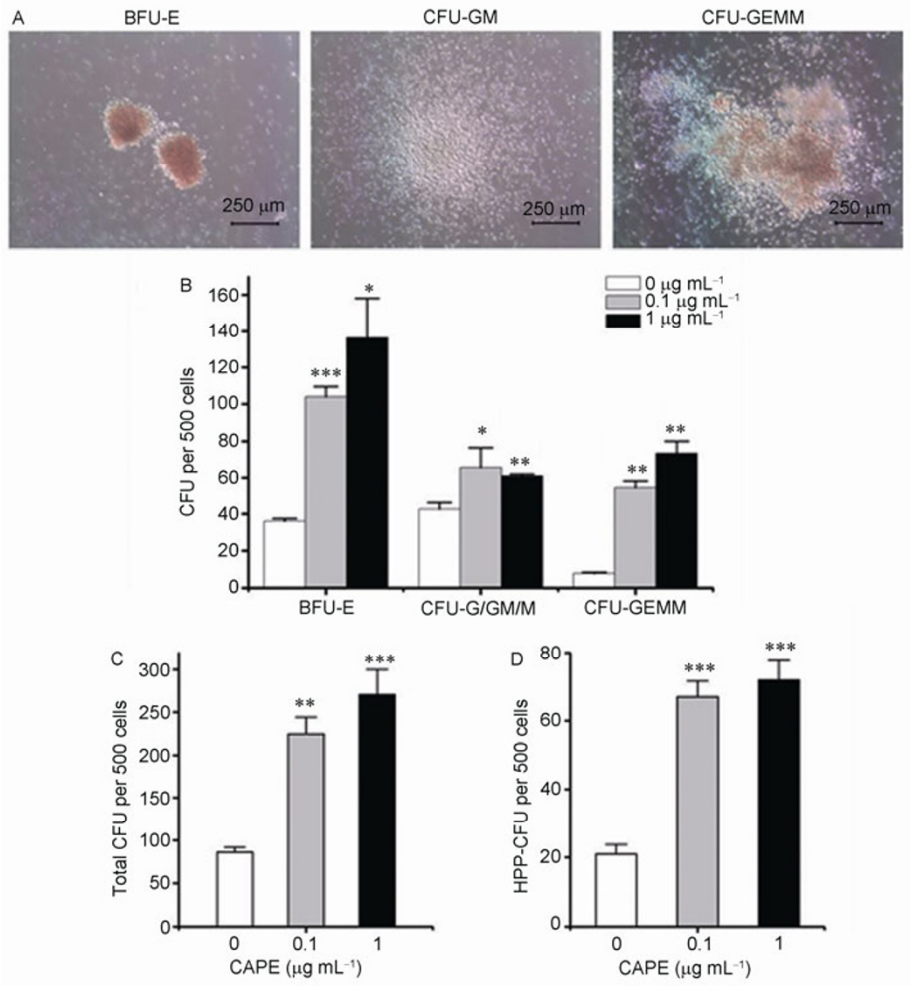

Figure 4 The CFU assays of $CD34^+$ cells cultured for 7 d at different CAPE concentrations. A, The colony morphology of BFU-E, CFU-GM and CFU-GEMM. B, The colony numbers of BFU-E, CFU-G/GM/M and CFU-GEMM. C, The total CFU numbers were calculated. D, The HPP-CFU numbers were counted. CFU-GM: colony forming unit-granulocyte and macrophage; CFU-G/GM/M: colony forming unit-granulocyte/granulocyte and macrophage/megakaryocyte. *, $P \leqslant 0.05$; **, $P \leqslant 0.01$ vs. 0 μg mL^{-1}.

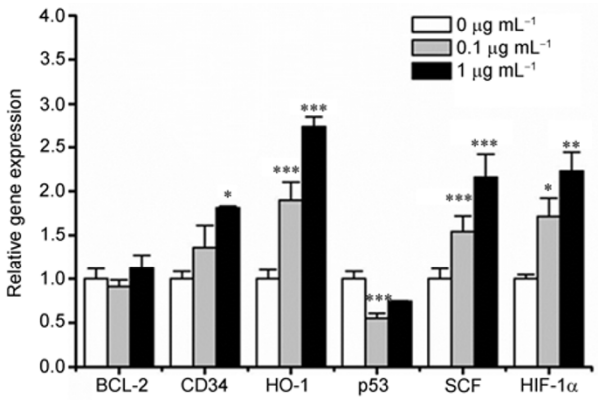

Figure 5 Quantitative PCR analysis of the expression levels of proliferation and apoptosis-related genes in MNCs cultured for 7 days with different concentrations of CAPE. *$P \leqslant 0.05$; **, $P \leqslant 0.01$ vs. 0 μg mL^{-1}.

activate the HIF-1 target genes, such as vascular endothelial growth factor and heme oxygenase-1 (HO-1), which play a protective role in I/R injury [21]. By using gene chip analysis, Wang reported that the expression levels of SCF and HO-1 genes were significantly upregulated in HUVECs. These genes play critical roles in HSPC proliferation and survival. Thus, we postulated that CAPE might function on HSPCs. We first evaluated its role on the survival of UCB MNCs. CAPE at low concentration levels (0.1 μg mL^{-1} or 1 μg mL^{-1}) showed little or slight effect on the survival rate of total nuclear cells. When the concentration of CAPE in medium reached 5 μg mL^{-1}, it induced the reduction of the total cell numbers compared to the control group (data not shown). CAPE treatment at low concentration (0.1 μg mL^{-1} and 1 μg mL^{-1}) increased the numbers of $CD34^+$ or $CD34^+CD38^-$ cell in cultured UCB MNCs. The similar results were also seen in CFU assays (data not shown). These data suggested that CAPE might be used as a supplement in HSPC culture medium. We then isolated UCB $CD34^+$ cells and cultured them in medium with cytokines plus CAPE at different concentrations. Similarly, low concentration of

CAPE (0.1 μg mL^{-1}) did not affect the apoptosis rate of cultured $CD34^+$ cells. However, CAPE at 5 μg mL^{-1} showed low cytotoxicity in cultured hematopoietic cells (data not shown). It has been reported that CAPE exerted the maximum cytoprotection in HUVECs against menadione-caused oxidative injury at 5 μg mL^{-1} and it produced cytotoxicity at 15 μg mL^{-1} [10]. These data suggested that different kinds of cells responded to different levels of CAPE in different ways. On the other hand, CAPE might increase the survival rate of cultured $CD34^+$ cells under stress. In a normal culture condition, CAPE-treated $CD34^+$ cells exhibited increased colony-forming capacity. Interestingly, BFU-E was the most obviously affected colony type, increasing more than 3.7-fold in cells treated with 1 μg mL^{-1} CAPE compared with the control group. We found that some genes (SCF, HIF-1α and HO-1) are significantly upregulated by CAPE in cultured UCB cells, which might be the mechanism for CAPE to expand HSPCs. SCF, the most important cytokine for HSPC self-renewal, therefore is crucial to UCB HSPC expansion *ex vivo*. HIF-1α, a transcription factor, plays a pivotal role in cellular adaption to hypoxia. It has been reported that hypoxia and upregulation of HIF-1α expression enhanced the proliferation of UCB HSPCs *in vitro* [22]. HO-1, a target gene of HIF-1α, also exerts the important role in the regulation of HSPC function. However, more work need to be done to further evaluate the function of CAPE-treated HSPCs, especially under stress conditions. For example, Wang's work showed that modification of the catechol ring of CAPE by introducing fluorine at various positions resulted in dramatic changes in cytoprotective activity [23]. Thus, it might be worth evaluating the role of CAPE derivatives on HSPC expansion under normal or stress culture condition.

In conclusion, CAPE, the small molecule from honeybee extract, can promote HSPC expansion *in vitro*. The mechanism of CAPE-induced HSPC expansion appears to involve upregulation of the expression of SCF, HIF-1α, and HO-1. Our data suggest that CAPE might become a new and potent medium supplement for *in vitro* HSPC expansion.

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Supporting Information

Table S1 Primer list

The supporting information is available online at life.scichina.com and link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.