

脐血间充质干细胞的体外培养及其表面标志变化*☆

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In vitro culture and surface marker variations of umbilical cord blood mesenchymal stem cells

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Abstract

BACKGROUND: Currently, there is not a standard method for *in vitro* culture and large scale amplification of umbilical cord blood mesenchymal stem cells (UCB-MSCs).

OBJECTIVE: To investigate the isolation, purification and culture of UCB-MSCs *in vitro*, and to detect its surface marker variation.

METHODS: The monocytes were harvested from UCB using 1.077 g/cm³ lymphocytes separating solution and density gradient centrifugation, followed by incubation in an incubator containing 5%CO₂ at 37 °C. The cell morphological changes were observed at different time points and the expression of surface marker was detected using flow cytometry.

RESULTS AND CONCLUSION: The monocytes isolated from the UCB grew initially into numerous hematopoietic cell clones, most of which were granulocyte/macrophage colony-forming units and burst forming unit-erythroid, increasing by (37.1±2.3) and (10.4±1.7), respectively. Switzerland staining showed most of them were granulocyte clones (80.1±85.2)%, next was erythroid clones (14.2±1.8)%. At 7 days after culture, some shuttle fibroblast-like cells and flat osteogenic-like cell spread the whole plastic well. At 14 days after culture, flow cytometry showed CD38⁺ cells accounted for 1.64%, and CD34⁺/CD38⁺ cells accounted for 1.71%, and CD34⁺/CD38⁻ were 0.55%. PI⁺ and Annexin-V⁺ cells accounted for 0.05% and 0.18% respectively. At 21 days after culture, CD38⁺, CD34⁺/CD38⁺ and CD34⁺/CD38⁻ cells were 74.32%, 1.61%, and 0.24%. The results revealed that UCB-MSCs can be isolated and cultured *in vitro*.

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摘要

背景: 目前有关脐血间充质干细胞的体外培养和大规模扩增方法不一, 存在一定困难。

目的: 观察脐血间充质干细胞体外分离和培养的方法, 并检测其表面分子的变化。

方法: 取新鲜采集脐带血, 用 1.077 g/cm³ 的淋巴细胞分层液, 密度梯度离心法分离脐血单个核细胞。将脐血单个核细胞接种于 37 °C、含体积分数为 5%CO₂ 培养箱内培养。于不同时间观察细胞形态的变化并通过流式细胞仪检测细胞表面分子的表达情况。

结果与结论: 从脐血中分离出的单个核细胞, 培养中先出现大量的造血细胞集落, CFU-GM 与 BFU-E 集落形成最多, 集落分别增加了 (37.1±2.3) 和 (10.4±1.7) 倍, 瑞氏染色显示这些细胞大多数为粒系的集落 (80.1±85.2)%, 其次为红系的细胞集落 (14.2±1.8)%, 7 d 后出现贴壁的扁平状上皮样细胞和长梭形成纤维样细胞, 同时有大量的破骨样细胞混杂。扩增后第 14 天经流式细胞仪分析 CD38⁺ 细胞为 1.64%, CD34⁺/CD38⁺ 细胞为 1.71%, CD34⁺/CD38⁻ 细胞为 0.55%, PI⁺ 细胞为 0.05%, Annexin-V⁺ 细胞为 0.18%。随着培养时间的延长, 细胞数目不断增加, 培养 21 d 时, 单个核细胞扩增了近 7.8 倍, 第 28 天增加了 1.71 倍。经流式细胞仪分析 CD38⁺ 细胞为 74.32%, CD34⁺/CD38⁺ 细胞为 1.61%, CD34⁺/CD38⁻ 细胞为 0.24%。提示脐血间充质干细胞可以体外培养。

关键词: 体外培养; CD34; CD38; 脐血; 间充质干细胞;

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0 引言

干细胞是具有自我更新能力和高度增殖能力, 以及多种分化潜能的较原始细胞, 在一定的条件下, 干细胞可以分化成机体内的多种功能细胞。脐带血中含有造血干细胞和间充质干细胞(mesenchymal stem cells, MSCs)。脐血造血干细胞通常被用作骨髓移植的供体细胞, 这些细胞能够产生各类造血细胞恢复造血功能。脐血间充质干细胞是一类来源于中胚层

的具有高度自我更新能力和多向分化潜能的成体干细胞, 其在临床上具有更广泛的应用前景。但是目前关于脐血间充质干细胞的体外培养方法不一, 获得较多数量的MSCs仍有困难。本实验中探讨了脐血MSCs体外分离和培养的方法, 并检测其表面分子的变化。

1 材料和方法

设计: 观察性实验。

时间及地点: 实验于2008-06/2009-06在郑

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州大学完成。

材料: 在产妇知情同意的情况下, 经医院伦理委员会批准, 获取年龄在24~35岁间的足月妊娠健康产妇脐血(郑州大学第三附属医院产科提供), 无肝炎、结核等各种妊娠合并症。

主要试剂及仪器:

试剂及仪器	来源
胎牛血清	杭州四季青
HEPES、表皮生长因子、干细胞因子	Sigma
H-DMEM培养基	Gibco
淋巴细胞分层液(密度为1.077 g/cm ³)	天津血液病研究所
碱性成纤维细胞生长因子	深圳晶美生物
OLMPUS IX71倒置显微镜	日本
流式细胞仪	美国

实验过程:

脐血间充质干细胞的分离与培养: 待新生儿娩出后, 立即断脐, 消毒母侧脐带断端, 60 mL 无菌注射针穿刺脐静脉取血, 立即导入肝素抗凝的无菌采血袋内(肝素抗凝终浓度为20~30 U/mL)。病原体检查为阴性, 无凝溶血现象。取新鲜采集脐带血(<15 h, 最好<4 h), 无菌PBS(pH7.4)等体积稀释, 混匀。用1.077 g/cm³的淋巴细胞分层液, 密度梯度435 g离心30 min分离脐血单个核细胞, 小心吸取中间白膜层, 加入2倍体积PBS, 1 000 r/min离心10 min洗涤2次, 彻底弃上清。H-DMEM培养液定容后锥虫蓝染色, 光学显微镜下计数活细胞。1.0×10¹¹ L⁻¹密度接种于6孔培养板内, 培养液为含体积分数为20%胎牛血清、200 μg/L干细胞因子, 置37 °C、体积分数为5%的CO₂饱和湿度培养箱内培养。培养过程中, 用倒置显微镜观察细胞形态的变化, 第一次3 d后全量换液, 以后每7 d换液1次。

流式细胞仪检测: 分别取新鲜和融合状态的脐血间充质干细胞, 去掉培养液, PBS洗2遍, 用37 °C温浴的2.5 g/L胰蛋白酶和0.2 g/L EDTA混合液(1:1)消化, 用含20 g/L BSA的PBS洗涤后制成浓度为1.0×10⁹ L⁻¹的单细胞悬液, 每个Eppendorf管加100 μL细胞悬液, 离心沉淀后加入红细胞裂解液, PBS离心洗涤后, 分别加入各种抗体。其中两管为阴性对照, 其余4管分别加入抗人的5 μL CD34 FITC、5 μL CD38PE、5 μL PI FITC、5 μL Annexin-V PE。室温孵育 20 min, 流式细胞仪检测。

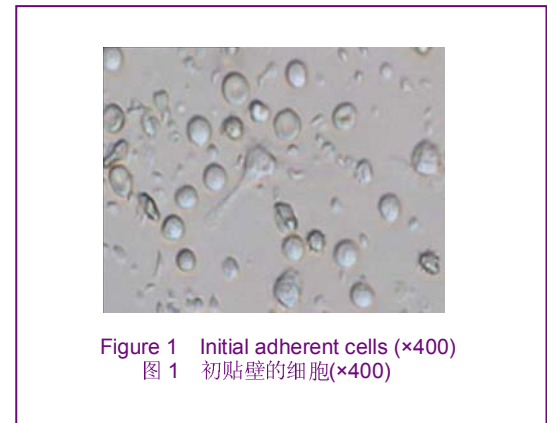
主要观察指标: 不同时间细胞的形态变化;

流式细胞仪检测细胞表面分子的表达。

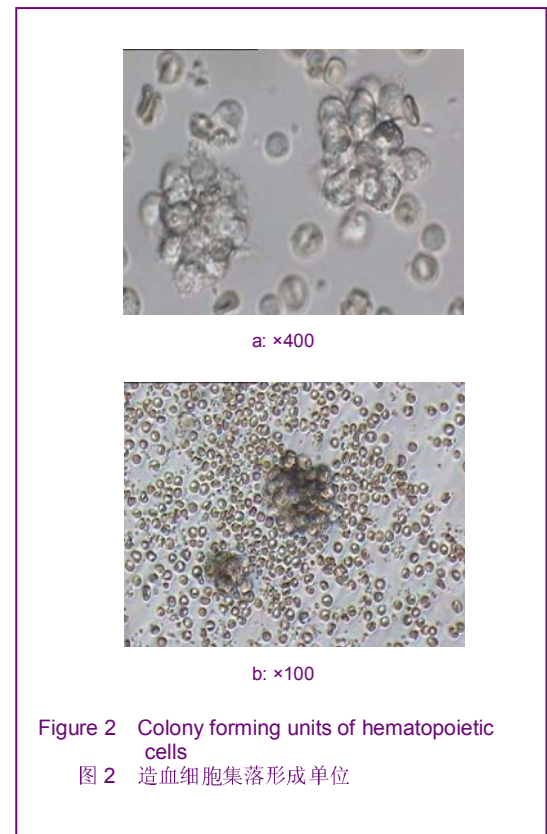
设计、实施、评估者: 实验设计及评估为第一作者和通讯作者, 干预实施为第二、三、四作者, 经过正规培训, 采用盲法评估。

2 结果

2.1 脐血间充质干细胞的形态变化 从脐血中分离出的单个核细胞, 胞体小呈圆形, 培养的第2天, 显微镜下可见少量贴壁细胞(见图1)。



3 d后出现大量的造血细胞集落(见图2)。



7 d后出现贴壁的扁平状上皮样细胞和长梭形的成纤维样细胞, 同时有大量的破骨样细

胞混杂(见图3, 4)。

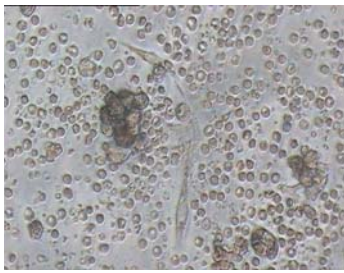


Figure 3 Shuttle-shaped cells could be seen at 7 days after culture (x100)
图3 培养7 d后出现梭形的细胞(x100)

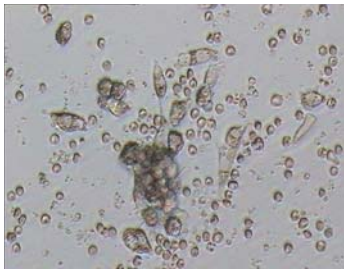


Figure 4 Proliferative shuttle-shaped cells (x100)
图4 大量增殖的梭形细胞(x100)

初次形成的破骨样细胞贴壁层由形态学上异质性细胞群构成, 镜下观察细胞呈细长、卵圆或圆形, 边缘完整, 其最显著的形态学特征为出现多核细胞, 而且这些核集中出现在细胞中心区, 破骨样细胞胞体较大, 多个核, 圆形(见图5)。



Figure 5 Osteogenic-like cells (x100)
图5 破骨样细胞(x100)

间充质干细胞为较均一成纤维样的长梭形细胞, 单个核, 有较长的突起, 折光性强, 细胞的胞浆清晰明亮, 所含的空泡或颗粒甚少, 形态类似于骨髓间充质干细胞, 但较骨髓间充质干细胞稍小(见图6)。

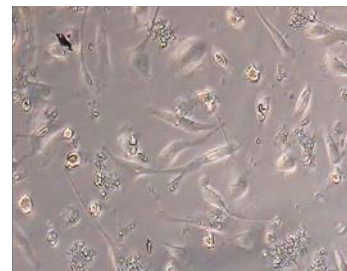


Figure 6 Long shuttle-shaped cells (x400)
图6 长梭形细胞(x400)

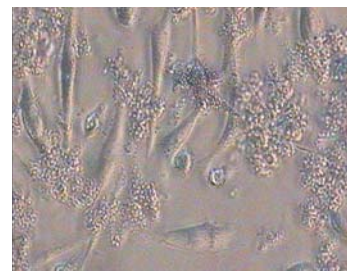
随着在条件培养基的环境下培养, 有少量细胞形态类似于树突状细胞(见图7), 大多数细胞呈现梭形, 四周后细胞生长旺盛(见图8), 可进行诱导实验。



Figure 7 Dendrite-like cells (x400)
图7 树突状细胞(x400)



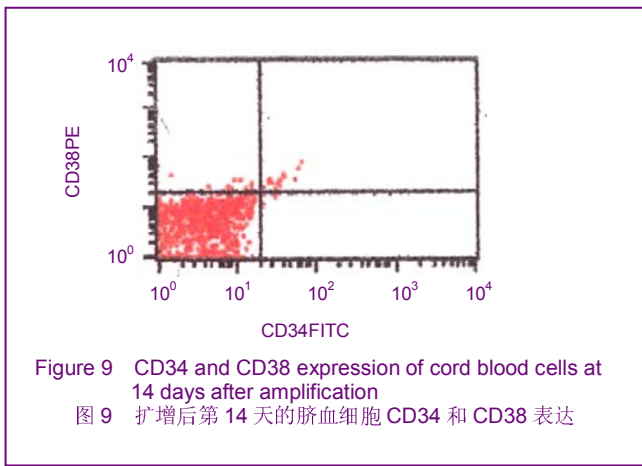
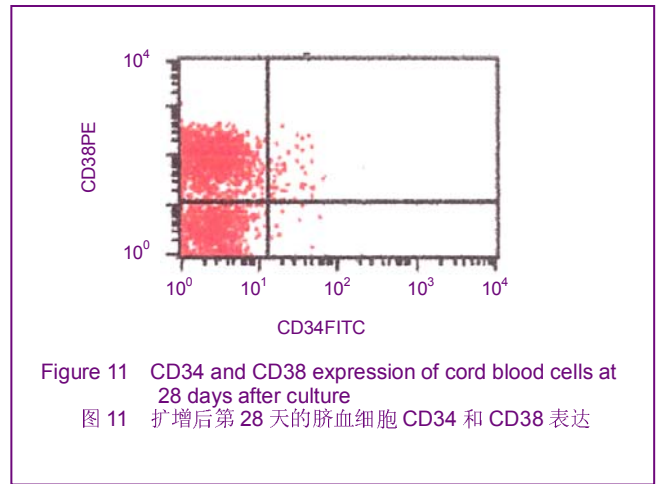
a: x100



b: x200

Figure 8 High proliferation of mesenchymal stem cells
图8 增殖旺盛的间充质干细胞

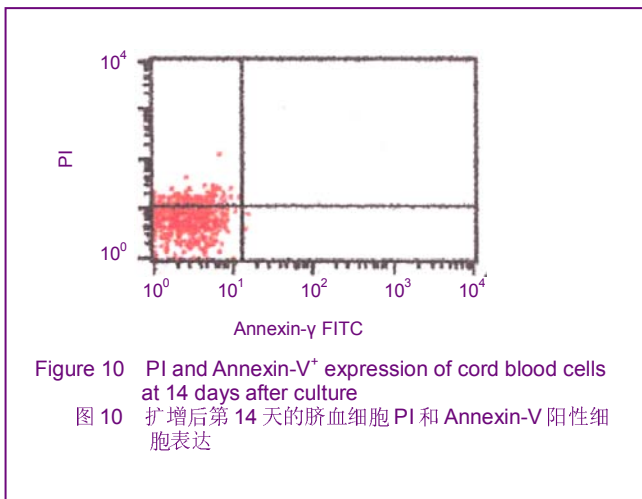
2.2 脐血间充质干细胞培养过程中表面标志的变化
 培养过程中先出现了造血细胞的集落, 扩增7 d时的脐血细胞, CFU-GM与BFU-E集落形成均最多; 细胞团大于50 μm, 集落分别增加了(37.1±2.3)和(10.4±1.7)倍, 瑞士染色显示这些细胞大多数为粒系的集落(80.1±85.2)%, 其次为红系的细胞集落(14.2±1.8)%, 扩增后第14天的脐血细胞CFU-GM、BFU-E集落数均低于培养7 d脐血单个核细胞(mononuclear cells, MNC)形成的集落数, 经流式细胞仪分析CD38⁺细胞为1.64%, CD34⁺/CD38⁺细胞为1.71%, CD34⁺/CD38⁻细胞为0.55%(见图9)。



而此时细胞几乎没有凋亡, PI⁺细胞为0.05%, Annexin-V⁺细胞为0.18%(见图10)。

3 讨论

间充质干/祖细胞是一群存在于造血系统内的非造血细胞, 1982年由Fridenshtein等^[1]发现并建立了以贴壁培养为主要手段的分离扩增方法。本实验在以往实验基础上^[2-3], 通过改进分离较多的脐血单个核细胞, 并培养出融合状态的间充质细胞。脐血间充质细胞的培养主要取决于以下几个方面: ①密度梯度离心的转速要严格控制, 且离心时间以30 min为宜, 这样白膜层较为清晰。②单个核细胞分离时间应该在采集脐带血后15 h内进行, 最好在4 h内完成分离。③每次分离脐带血的量要多于33 mL。④分离得到的单个核细胞数量大于1×10⁸个/cm²。⑤采集的脐带血无凝血或溶血现象。⑥脐血细胞在培养过程中需要用塑料培养板, 这样可以增加细胞的贴壁数量。⑦培养基中需要加入一些细胞因子, 本实验中加入了碱性成纤维细胞生长因子, 干细胞因子促进细胞的增殖。这些细胞因子可以使G₀期细胞启动进入G₁期, 从而发挥其增殖潜能。



脐血细胞在培养过程中出现了一些形态学和表面分子表达的变化^[4-7]。从脐血中分离出的单个核细胞, 培养后首先出现大量的造血细胞集落, 而后才出现间充质细胞的生长^[8]。由于脐血中成分复杂, 分离过程中很难将造血细胞去除^[9-11], 因此出现了早期培养中造血细胞的克隆。CD34是造血干细胞的表面标志, 造血干细胞体外扩增后, 评价指标主要包括总有核细胞的扩增和CD34⁺细胞的扩增。Koller等^[12-15]在连续灌注生物反应器内培养骨髓来源的单核细胞2周, 有核细胞、粒-单系集落形成单位、红系爆式集落形成单位及长期培养起始细胞均有扩增。本实验中通过观察CD34和CD38等标志, 观察并评价本实验体系。实验结果显示CD34⁺细胞数量随着培养时间的延长而减少, 这与形态学的变化是相吻合的。Yang等^[16-18]采用在a-MEM培养基中添加体积分数为10%胎牛血清, 培养MSCs, 在411份脐血当中

有95份培养出了MSCs, 占总数的23%。本实验室通过增加干细胞因子等, 增加了MSCs的存活率。

近年来, 细胞凋亡的研究引起了人们广泛的重视^[19-25]。碘化丙啶染色法根据光散射的特点, 可使凋亡细胞与坏死细胞区别开来。在DNA凋亡细胞峰图上, 凋亡细胞出现二倍体峰(G₁细胞)的减少^[26-31]。Annexin-V是一种Ca²⁺依赖性磷脂结合蛋白, 凋亡早期, 细胞内膜的磷脂酰丝氨酸移位到细胞膜外侧, 可以与荧光素标记的Annexin-V结合, 采集不同的标记荧光, 输入计算机, 分析打印出散点图。正常情况下活细胞不会被染色, 凋亡细胞可被标记上Annexin-V, 坏死和凋亡晚期细胞可被Annexin-V和碘化丙啶同时染色。实验中细胞凋亡较少, 提示所培养的细胞增殖能力较强, 适合于做进一步的研究。

4 参考文献

- [1] Fridenshtein AT. Stromal bone marrow cells and the hemato-poietic micro-environment. *Arkh Pathol.* 1982;44(10):3.
- [2] Urciuoli P, Passeri S, Ceccarelli F, et al. Pre-birth selection of umbilical cord blood donors. *Blood Transfus.* 2010;8(1):36-43.
- [3] Lemarie C, Bouchet G, Sielleur I, et al. A new single-platform method for the enumeration of CD34+ cells. *Cytotherapy.* 2009;11(6):804-806.
- [4] Wang TY, Chang SJ, Chang MD, et al. Unique biological properties and application potentials of CD34+ CD38- stem cells from various sources. *Taiwan J Obstet Gynecol.* 2009;48(4):356-369.
- [5] Stojko R, Witek A, Glogowska-Lingus J, et al. Transcriptional activity of telomerase complex in CD34- stem cells of cord blood in dependence of preparation time. *Folia Histochem Cytobiol.* 2009;47(2):265-274.
- [6] Takahashi K, Monzen S, Hayashi N, et al. Correlations of cell surface antigens with individual differences in radiosensitivity in human hematopoietic stem/progenitor cells. *Radiat Res.* 2010;173(2):184-190.
- [7] Schlechta B, Wiedemann D, Kittinger C, et al. Ex-vivo expanded umbilical cord blood stem cells retain capacity for myocardial regeneration. *Circ J.* 2010;74(1):188-194.
- [8] Liu SF, Li CS, Xing Y, et al. Morphological observation of pancreatic stem cells and cord blood mesenchymal stem cells cultured in vitro. *Zhongguo Linchuang Kangfu.* 2006;10(9):183-185.
- [9] Shahrokhi S, Ebtekar M, Alimoghaddam K, et al. Substance P and calcitonin gene-related neuropeptides as novel growth factors for ex vivo expansion of cord blood CD34(+) hematopoietic stem cells. *Growth Factors.* 2010;28(1):66-73.
- [10] Pranke P, Hendrikx J, Debnath G, et al. Immunophenotype of hematopoietic stem cells from placental/umbilical cord blood after culture. *Braz J Med Biol Res.* 2005;38(12):1775-1789.
- [11] Park CY, Majeti R, Weissman IL. In vivo evaluation of human hematopoiesis through transplantation of purified hematopoietic stem cells from umbilical cord blood. *Nat Protoc.* 2008;3(12):1932-1940.
- [12] Koller MR, Elnerson SG, Palsson BO. Large scale expansion of human stem cells from bone marrow mononuclear cells in continuous perfusion cultures. *Blood.* 1993;82(2):378-384.
- [13] Turnovcova K, Ruzickova K, Vaneczek V, et al. Properties and growth of human bone marrow mesenchymal stromal cells cultivated in different media. *Cytotherapy.* 2009;11(7):874-885.
- [14] Singh K, Srivastava A, Mathur N, et al. Evaluation of four methods for processing human cord blood and subsequent study of the expansion of progenitor stem cells isolated using the best method. *Cytotherapy.* 2009;11(6):768-777.
- [15] Flores-Guzmán P, Flores-Figueroa E, Montesinos JJ, et al. Individual and combined effects of mesenchymal stromal cells and recombinant stimulatory cytokines on the in vitro growth of primitive hematopoietic cells from human umbilical cord blood. *Cytotherapy.* 2009;11(7):886-896.
- [16] Yang SE, Ha CW, Jung M, et al. Mesenchymal stem /progenitor cells developed in cultures from UC blood. *Cytotherap.* 2004;6(5):476-486.
- [17] Araki H, Baluchamy S, Yoshinaga K, et al. Cord blood stem cell expansion is permissive to epigenetic regulation and environmental cues. *Exp Hematol.* 2009;37(9):1084-1095.
- [18] Delalat B, Pourfathollah AA, Soleimani M, et al. Isolation and ex vivo expansion of human umbilical cord blood-derived CD34+ stem cells and their cotransplantation with or without mesenchymal stem cells. *Hematology.* 2009;14(3):125-132.
- [19] Mastino A, Favalli C, Camilli AR, et al. Umbilical cord blood: the role of apoptosis in the control of CD34+ cell counts. *Placenta.* 2003;24(1):113-115.
- [20] Hiwase SD, Dyson PG, To LB, et al. Cotransplantation of placental mesenchymal stromal cells enhances single and double cord blood engraftment in nonobese diabetic/severe combined immune deficient mice. *Stem Cells.* 2009;27(9):2293-2300.
- [21] Knaän-Shanzer S, van der Velde-van Dijke I, van de Watering MJ, et al. Phenotypic and functional reversal within the early human hematopoietic compartment. *Stem Cells.* 2008;26(12):3210-3217.
- [22] Fujita S, Toguchida J, Morita Y, et al. Clonal analysis of hematopoiesis-supporting activity of human mesenchymal stem cells in association with Jagged1 expression and osteogenic potential. *Cell Transplant.* 2008;17(10-11):1169-1179.
- [23] Matsumoto K, Terakawa M, Nakajima T, et al. Extremely rapid and intense induction of apoptosis in human eosinophils by anti-CD30 antibody treatment in vitro. *J Immunol.* 2004;172(4):2186-2193.
- [24] Parant O, Dubernard G, Chailier JC, et al. CD34+ cells in maternal placental blood are mainly fetal in origin and express endothelial markers. *Lab Invest.* 2009;89(8):915-923.
- [25] Geest CR, Zwartkruis FJ, Vellenga E, et al. Mammalian target of rapamycin activity is required for expansion of CD34+ hematopoietic progenitor cells. *Haematologica.* 2009;94(7):901-910.
- [26] Steiner D, Gelovani J, Savoldo B, et al. Noninvasive bioluminescent imaging demonstrates long-term multilineage engraftment of ex vivo-expanded CD34-selected umbilical cord blood cells. *Stem Cells.* 2009;27(8):1932-1940.
- [27] Rollini P, Faes-Van't Hull E, Kaiser S, et al. Phenotypic and functional analysis of human fetal liver hematopoietic stem cells in culture. *Stem Cells Dev.* 2007;16(2):281-296.
- [28] McKenzie JL, Gan OI, Doedens M, et al. Reversible cell surface expression of CD38 on CD34-positive human hematopoietic repopulating cells. *Exp Hematol.* 2007;35(9):1429-1436.
- [29] Christopherson KW, Uralil SE, Porecha NK, et al. G-CSF- and GM-CSF-induced upregulation of CD26 peptidase downregulates the functional chemotactic response of CD34+CD38- human cord blood hematopoietic cells. *Exp Hematol.* 2006;34(8):1060-1068.
- [30] McKenzie JL, Gan OI, Doedens M, et al. Individual stem cells with highly variable proliferation and self-renewal properties comprise the human hematopoietic stem cell compartment. *Nat Immunol.* 2006;7(11):1225-1233.
- [31] Paczesny S, Li YP, Li N, et al. Efficient generation of CD34+ progenitor-derived dendritic cells from G-CSF-mobilized peripheral mononuclear cells does not require hematopoietic stem cell enrichment. *J Leukoc Biol.* 2007;81(4):957-967.

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