· ARTICLES · ①

· 论 著 ·

Two conditional media promoting the differentiation murine embryonic stem cells into hematopoietic stem cells

ZHAO Hui-ping[1], LU Guang-xiu[1], WANG Qi-ru[1,2]

(1. *Institute of Human Reproductive and Stem Cell Engineering, Central South University, National Human Stem Cell Engineering Research Center, Changsha* 410078; 2. *Department of Blood Laboratory, Xiangya School of Medical, Central South University; Changsha* 410078, *China*)

Abstract: Objective To derive hematopoietic stem cells with functional properties of hematopoietic reconstitution from murine embryonic stem (ES) cells. **Methods** ES-D3 cells by formation of the day-4 embryoid bodies (4dEBs) were induced into hematopoietic stem cells by co-culture with murine bone marrow endothelial cell-conditional medium (mBMEC-CM) and the fetal liver stromal cell-conditional medium (FLSC-CM). This experiment was designed to 4 groups (mBMEC-CM + FLSC-CM group, mBMEC-CM group, FLSC-CM group, and the control group). **Results** The total cell numbers, $CD34^+$ cell numbers, and colony numbers formed in the mBMEC-CM + FLSC-CM group were the highest among the 4 groups. The cells in the mBMEC-CM + FLSC-CM group resumed the hematopoietic system of the mice after being transplanted with the inducing cells. **Conclusion** The culture condition combing mBMEC-CM with FLSC-CM can promote murine ES cells differentiating into hematopoietic stem cells with functional properties of hematopoietic reconstitution.

Key words: embryo, stem cells; embryoid body; hematopoiesis; hematopoietic stem cell; bone marrow, endothelial cell

[*J Cent South Univ (Med Sci)*, 2006,32(1):0063-06]

两种条件培养基促进鼠胚胎干细胞分化为造血干细胞

赵惠萍[1],卢光琇[1],王绮如[1,2]

(1. 中南大学生殖与干细胞工程研究所,人类干细胞国家工程研究中心,长沙 410078;
2. 中南大学湘雅医学院血液生理研究室,长沙 410078)

[**摘要**] 目的:从鼠胚胎干细胞获得具有造血重建功能的造血干细胞。方法:将 ES-D3 细胞形成 4 天拟胚体(4dEBs),再用骨髓内皮细胞条件培养液(mBMEC-CM)和/或胎肝基质细胞条件培养液(FLSC-CM)诱导 4dEBs 生成造血干细胞。实验为 mBMEC-CM + FLSC-CM 组、mBMEC-CM 组、FLSC-CM 组,对照组。检测诱导生成的细胞造血干细胞特异抗原表达、造血相关基因表达、造血集落的形成以及造血重建能力。结果:从诱导 ES-D3 细胞生成造血干/祖细胞的数量和生成的集落总数看,mBMEC-CM + FLSC-CM 组诱导效率显著高于其他 3 组。mBMEC-CM +

①**Date of reception** 2006-06-01

Biography ZHAO Hui-ping(1974-), female, Lecturer, Ph. D., mainly engaged in hematopoietic regulation.

Corresponding author WANG Qi-ru, E-mail: qirwang@yahoo.com

Foundation items This work was supported by National Natural Science Foundation of China (30300119 and30030070) and Research Fund for the Doctoral Program of Higher Education from the Ministry of Education of China (20030533002)

FLSC-CM 组诱导生成的细胞能重建照射鼠的造血系统。结论:骨髓内皮细胞条件培养液联合胎肝基质细胞条件培养液可促进鼠胚胎干细胞分化为具有造血重建能力的造血干细胞。

[关键词] 胚胎,干细胞; 拟胚体; 造血; 造血干细胞; 骨髓,内皮细胞

[中图分类号] R331.1 [文献标识码] A [文章编号] 1672-7347(2007)01-0063-06

Hematopoietic stem cells (HSCs) generated from embryonic stem (ES) cell differentiation culture are suitable for bone marrow transplantation and gene therapies. Primitive HSCs from ES cell differentiation culture can offer theoretic therapeutic advantages to adult bone marrow HSCs, including more extended proliferative capacity, developmental potential and less propensity to cause graft-versus-host disease[1].

In studies of hematopoietic differentiation from ES cells, the main methods are to co-culture ES cells with the murine fibroblast lines such as OP9, RP0.10, MS-5, NIH-3T3, PA6[2-7]. There are few studies on bone marrow endothelial cell line of inducing ES cell differentiation into hematopoietic cells. Recently, we reported that murine bone marrow endothelial cell-conditional medium (mBMEC-CM) and/or a combination of cytokines can promote murine ES cell differentiation to hematopoietic precursor cells[8]. In this report, we detected the ability of murine ES cell-derived HSCs of long-term reconstitution of the hematopoietic system in recipients by co-culturing murine ES cells with mBMEC-CM and FLSC-CM.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Murine ES Cell Line and Animals The murine ES cell line ES-D3 and the nude mice (6-week-old females, BALB/C) were provided by professor Cheng Xi-gu in Department of Laboratory Animal Science, Sun-Yat-Sen University.

1.1.2 Reagents Fetal bovine sera (FBS) were purchased from Hyclone Company. Horse sera were purchased from Academy of Military Medical Sciences. Cytokines including leukemia inhibitory factor (LIF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), erythropoietin (EPO), recombinant interleukin (rIL)-3, rIL-6 and Trizol reagents were products of GIBCO/BRL. L-glutamine (L-G), methylcellulose, agar and bovine serum albumin (BSA) were products of Sigma. Purified rat anti-mouse Sca-1(Clone D7), c-kit(Clone 2B8), CD11b(Clone M1/70), Erythoid cell marker(Clone TER-119) monoclonal antibodies were the products of eBioscience Company and purified rat anti-mouse CD34 (Clone MEC 14.7) monoclonal antibody was the product of Hycult biotechnology Corporation. Purified rat anti-mouse Thy-1 (Clone IBL-1) monoclonal antibody was the product of Neomarkers Corporation. Phycoprobe R-Phycoerythrin (PE) Anti-Rat IgG (H + L spec.) was purchased from Biomeda Corporation. Immunocytochemistry kit and DAB kit were purchased from WuHan Boster Biological Technology Co. LTD. RT-PCR kit was purchased from TaKaRa Biotechnology (Dalian) Co. LTD. Primers were synthesized by Shanghai sangon Biotechnology Co. LTD.

1.2 Methods

1.2.1 Culture of ES cells The murine ES cell line ES-D3 was derived and maintained in the undifferentiated state as described[9]. We detected the ES cell line ES-D3 undifferentiated characters of alkaline phosphatase, karyotype, and the ability of differentiation in vitro and in vivo before the following experiments. The ES cells were maintained as undifferentiated cells by culture on monolayers of mitomycin C-treated (10 μg/mL at 37 ℃ for 4 hours) murine embryonic fibroblast (MEF) cells.

1.2.2 Collection of mBMEC-CM and FLSC-CM The cell line SC_{95} was established in our lab[9]. The fetal liver stromal cells were prepared as described[3]. When they grew confluent, the SC_{95} cells and the fetal liver stromal cells were cultured with serum-free Iscove' Modified Dubecco's Medium (IMDM, 0.1 mL/cm^2 culture container) for 48 hours after washed with saline 3 times respectively. Then the culture media were collected and centrifuged. The super-

natants were reserved in −20 ℃ after passed through 0.22 μm nitrocellulose filter units.

1.2.3 **Promoting ES cell differentiation to hematopoietic precursor cells** The day-4 Embryoid Bodies (4dEBs) were formed, collected and then replated in 2 mL culture medium (1×10^5/mL) by being divided into 4 groups (mBMEC-CM + FLSC-CM group, mBMEC-CM group, FLSC-CM group and control group). The concentrations of mBMEC-CM and FLSC-CM were 20% (vol/vol) respectively. These cells were harvested after 3 days. The detections of hematopoietic colony assay, flow cytometry analysis, immunocytochemistry and RT-PCR analysis were done according to the methods described in the paper[8].

1.2.4 **Hematopoietic reconstitution of irradiated recipient mice** Fifteen nude female mice were injected intravenously (lateral tail vein) after being given the dose of 350 cGy γ-irradiation. Eight out of fifteen mice received 2×10^6 mononuclear cells generated from the ES cell-derived cells of mBMEC-CM + FLSC-CM group induced for 3 days and seven out of fifteen mice received no cells in PBS as control. Transplanted mice were maintained under sterile conditions. Three months later, the bone marrow hematopoietic colonies were determined for myeloid differentiation. The presences of the Sry male gene in the colony cells and the cells obtained from the spleen or bone marrow of reconstituted female nude mice hosts were assessed by DNA-based PCR assays. The forward primer was 5′-CTGCTGTGAACAGACACTAC-3′; and the reverse primer was 5′-GACTCCTCTGACTTCACTTG-3′. And the length of PCR product of the Sry gene was 722 bp. The expressions of β-H_1 gene mRNA were detected in spleen cells of the experimental mice and the control mice by RT-PCR.

1.3 **Statistical Analysis** The data were presented as $\bar{x} \pm s$ and analyzed by SPSS statistical software (version 13.0). One-factor analysis of variance was applied to assess significances among groups and q-test was applied to assess significances between 2 groups. The statistical significance level was set at a two-tailed value of $P < 0.05$.

2 RESULTS

2.1 **Characters of the undifferentiated ES-D3 cells** The ES-D3 cells were positive to alkaline phosphatase staining, and the ES-D3 cell line had normal diploid karyotype. Forty chromosomes were found more than 60% of the cells (XY). In vitro the ES-D3 cells formed complex EBs with endoderm, mesoderm and ectoderm in suspension culture. In vivo the ES-D3 cells formed solid teratocarcinomas containing the products of three germ layers after being injected subcutaneously into syngeneic mice.

2.2 **Hematopoietic precursor cells derived from the ES cells** The results of hematopoietic progenitor assays showed that the ES cell-derived cells could form the hematopoietic colonies of HPP-CFC and BFU-E. In HPP-CFC culture, ES cell-derived colony cells had the same characteristic morphologies of granulocyte and macrophage as those from murine bone marrow cells. And most of these colony cells expressed antigen CD11b specially on macrophage, granulocyte and NK cell surface. In BFU-E culture, ES cell-derived colony cells were positive to benzidine, and most of these expressed erythroid specific antigen TER-119. Moreover these colony cells expressed adult (β-major) and embryonic (β-H1) globin gene mRNA, while undifferentiated ES cells did not express them.

RT-PCR results showed that the cells of the mBMEC-CM + FLSC-CM group and the mBMEC-CM group both expressed hematopoietic transcription factors (c-myb, SCL, and β-H1), while the cells of the FLSC-CM group and the control group only expressed *c-myb* gene.

The results of immunocytochemistry staining showed that ES cell-derived cells expressed c-kit, Sca-1, Thy-1, and CD34 antigens. The results of flow cytometry analysis demonstrated that the population of the mBMEC-CM + FLSC-CM group contained c-kit$^+$ cell 3.1%, Sca-1$^+$ cell 1.5%, Thy-1$^+$ cell 1.3%, while the population of the control group contained c-kit$^+$ cell 0.4%, Sca-1$^+$ cell 0%, Thy-1$^+$ 2.0%. The ratios of CD34$^+$ cells of the mBMEC-CM + FLSC-CM group and

the mBMEC-CM group were 69% and 62.5% respectively. Both were much higher than those of the FLSC-CM group and the control group (Fig. 1).

According to the total cell numbers, $CD34^+$ cell numbers, colony numbers (HPP-CFC and BFU-E), the effect of the mBMEC-CM + FLSC-CM group on promoting the generation of the ES cell-derived hematopoietic precursor cells was the strongest among the 4 groups (Table 1).

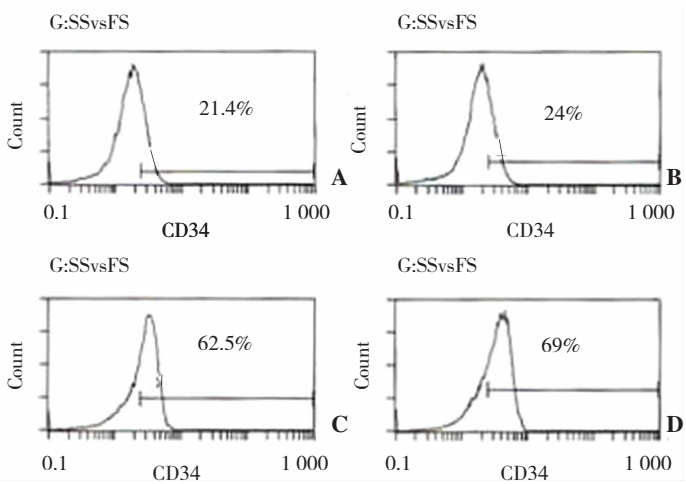

Fig. 1 Flow cytometry analysis of CD34 antigen on the cells from 4 groups

Table 1 Comparison of the effects of promoting hematopoietic differentiation from ES cells in the 4 groups

Group	cell number ($\times 10^4$)	$CD34^+$ number ($\times 10^4$)	HPP-CFC(number/10^5)	BFU-E(number/10^5)
mBMEC-CM + FLSC-CM group	$34.25 \pm 1.59^{**\#\#\triangle\triangle}$	$23.63 \pm 1.10^{**\#\#\triangle\triangle}$	$97.33 \pm 11.78^{**\#\#\triangle\triangle}$	$555.50 \pm 5.29^{**\#\#\triangle}$
mBMEC-CM group	$20.92 \pm 2.05^{**\#\#}$	$13.07 \pm 1.28^{**\#\#}$	$74.67 \pm 8.26^{**\#\#}$	$447.60 \pm 65.09^{**\#\#}$
FLSC-CM group	2.58 ± 0.70	0.62 ± 0.17	0.00	113.50 ± 21.97
Control group	3.50 ± 0.72	0.75 ± 0.15	0.00	119.83 ± 29.21

Compared with control group, $**$ $P<0.01$; Compared with FLSC-CM group, ## $P<0.01$; Compared with mBMEC-CM group, $\triangle$ $P<0.05$, $\triangle\triangle$ $P<0.01$

2.3 Hematopoietic reconstitution of irradiated recipient mice

2.3.1 Survival recipient mice Three months after transplantation, the number of survival mice in the experimental group was six out of eight and the number of survival mice in the control group was two out of seven.

2.3.2 Reconstitutions of hematopoietic lineage

The colony numbers of CFU-GM and BFU-E were 126.56 ± 13.88 and 65.4 ± 8.14 per 10^5 cells in the experimental group, and 33.50 ± 0.14 and 30.9 ± 5.90 per 10^5 cells in the control group. The colony numbers of CFU-GM and BFU-E of the experimental group were much higher than those of the control group ($P<0.01$).

There was *Sry* gene in these colony cells of the experimental mice while there was no *Sry* gene in those colony cells of control survival mice (Fig. 2).

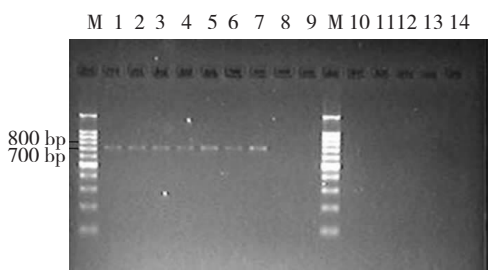

Fig. 2 PCR products of *Sry* male-specific gene (722 bp) Lane 1 to 6: CFU-GM cells, BFU-E cells, peripheral blood cells, thymocytes, bone marrow cells and spleen cells from female survival mouse ($5^\#$) that received the cells generated by induced ES cells. Lane 7: male normal mice; lane 8: female normal mouse; Lane 9 to 14: CFU-GM cells, BFU-E cells, peripheral blood cells, thymocytes, bone marrow cells and spleen cells from female survival mouse ($12^\#$) that received no cells. Lane M: marker

There was the expression of β-H_1 gene mRNA in spleen cells of experimental survival mice while there was no expression in the spleen cells of control survival mice (Fig. 3).

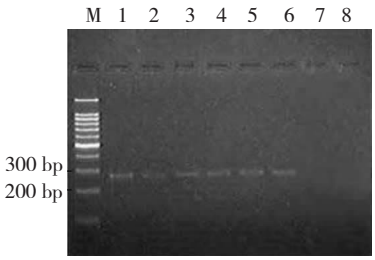

Fig. 3 RT-PCR products of β-H_1 gene (265bp) expression of spleen of survival animals after 3 months of transplantation Lane M: marker; Lane1-6: spleen cells of survival mice of experimental group; Lane7-8: spleen cells of survival mice of control group

3 DISCUSSION

Although ES cells can differentiate into all lineages of the blood system in vitro, efficient production of functional HSCs that can reconstitute all hematopoietic lineages in vivo has proven difficult[10]. In this study, we derived hematopoietic stem cells from murine ES cells by co-culture with murine bone marrow endothelial cell-conditional medium and the fetal liver stromal cell-conditional medium. The ES-D3 cell line was testified that the cells did not express *CD34* mRNA[11]. The increased long-term survival number of the experimental group of our culture conditions showed that these cells can exercise function of hematopoietic stem cell. We also employed hematopoietic clonal analysis of hematopoietic populations of engrafted mice to demonstrate our derivation of self-renewing, multipotential HSCs from murine ES cells. In this experiment, the increasing colony numbers (HPP-GM and BFU-E) and the existence of *Sry* gene showed that the increasing granulocyte, macrophage and erythroid cells are from exogenous HSCs derived ES-D3 cells. And the expressions of β-H_1 gene in the period of embryo on spleen cells also demonstrated that there are exogenous erythroid cells derived from ES-D3 cells. Thus, our culture conditions enable the directed differentiation of murine ES cells into hematopoietic progenitors with the cardinal features of definitive HSCs.

Stromal cells are major components of the microenvironment surrounding hematopoietic cells and play an important role in hematopoiesis in vivo. Co-culture of HSCs with stromal cells promoted hematopoiesis and self-renewal of HSCs[12-13]. It was reported that bone marrow stromal cell lines can induce ES cells differentiated to hematopoietic cells[2,7]. Fu et al.[14] also revealed that both AGM and BM stromal cells can promote the ESCs-derived hematopoietic precursor cells with the ability of hematopoietic reconstruction in vivo. In this study we used bone marrow endothelial cell line inducing ES cells differentiation into hematopoietic stem cells. The bone marrow endothelial cell line SC_{95} used in this experiment was a pure bone marrow endothelial cell line established in our lab[15]. In addition to hematopoietic stromal cells, combination of cytokines and special gene expressions are considered taking part in promoting hematopoietic differentiation from ESCs. Chadwick et al.[16] reported that the treatment of ESCs during EB development with a combination of cytokines and bone morphogenetic protein-4 (BMP-4) strongly promotes hematopoietic differentiation. Our previous results demonstrated positive and negative hematopoietic cytokines were produced by bone marrow endothelial cells[17]. So we infer that murine bone marrow endothelial cell-conditional medium contains these positive and negative hematopoietic cytokines which promotes hematopoietic differentiation from ESCs. These two conditional media of mouse bone marrow endothelial cells and the fetal liver stromal cells were used to gain ES-D3 cell-derived HSCs without contamination of stromal cells and can substitute for extensively exogenous hematopoietic cytokines.

In this study the cellular differentiating model was created by inducing ESCs differentiated into HSCs. In the following experiment we are going to explore which effects are exerted on the generation, maintenance, and expansion of HSCs from ES cells by murine bone marrow endothelial cell-conditional medium and the fetal liver stromal cell-conditional medium.

Acknowledgement: We thank professor Cheng Xi-gu and adjunct professor Huang-Bing of Department of Experimental Animal Institute, Sun-Yat-sen university for their guidance and kindly help.

References:

[1] Weiss M J. Embryonic stem cells and hematopoietic stem cell biology[J]. Hematol Oncol Clin North Am, 1997, 11(6):1185-1198.

[2] Gutierrez-Ramos J C, Palacios R. In vitro differentiation of embryonic stem cells into lymphocyte precursors able to generate T and B lymphocytes in vivo[J]. Proc Natl Acad Sci USA, 1992,89(19):9171-9175.

[3] Palacios R, Golunski E, Samaridis J. In vitro generation of hematopoietic stem cells from an embryonic stem cell line[J]. Proc Natl Acad Sci USA, 1995,92(16):7530-7534.

[4] Perkins A C. Enrichment of blood from embryonic stem cells in vitro[J]. Reprod Fertil Dev, 1998,10(7-8):563-572.

[5] Bigas A, Martin D I K, Bernstein I D. Generation of hematopoietic colony-forming cells from embryonic stem cells: Synergy between a soluble factor from NIH-3T3 cells and hematopoietic growth factors [J]. Blood, 1995(11),85:3127-3133.

[6] Berthier R, Prandini M H, Schweitzer A, et al. The MS-5 murine stromal cell line and hematopoietic growth factors synergize to support the megakaryocytic differentiation of embryonic stem cells[J]. Exp Hematol, 1997,25(6):481-490.

[7] Nakano T, Kodama H, Honjo T. Generation of lymphohematopoietic cells from embryonic stem cells in culture[J]. Science, 1994, 265(5262):1098-1101.

[8] Zhao Hui-ping, Lu Guang-xiu, Wang Qi-ru. Bone marrow endothelial cell-conditioned medium promotes hematopoietic differentiation of mouse embryonic stem cells[J]. J Chin Exp Hematol, 2003,11(2):109-114.

[9] Doetschman T C, Eistetter H, Kentler R, et al. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium[J]. J Embryol Exp Morph, 1985, 87:27-45.

[10] Wang Y, Frank Y, Olaia N, et al. Embryonic stem cell-derived hematopoietic stem cells[J]. PNAS, 2005, 102(52):19081-19086.

[11] Li F, Shen B J, Liu X X, et al. The promoting effect of vascular endothelial growth factor on the generation of CD34 cells from mouse embryonic stem cells[J]. Chin J Cancer Biother, 2004,11(3):191-194.

[12] Fry T J, Mackall C L. Immune reconstitution following hematopoietic progenitor cell transplantation: challenges for the future[J]. Bone Marrow Transplant, 2005,35 (Suppl 1):S53-S57.

[13] Krampera M, Pasini A, Rigo A, et al. HB-EGF/HER-1 signaling in bone marrow mesenchymal stem cells: inducing cell expansion and reversibly preventing multilineage differentiation[J]. Blood, 2005,106(1):59-66.

[14] Fu J R, Liu W L, Zhou Y F, et al. Expansive effects of aorta-gonad-mesonephros-derived stromal cells on hematopoietic stem cells from embryonic stem cells[J]. Chin Med J, 2005,118(23):1979-1986.

[15] Wang Q R, Yan Y, Wang B H, et al. Long-term culture of murine bone-derived endothelial cells[J]. In Vitro Cell Dev Biol, 1998, 34(6):443-446.

[16] Chadwick K, Wang L H, Li L, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells [J]. Blood, 2003, 102(3):906-915.

[17] Li W M, Huang W Q, Huang Y H, et al. Positive and negative hematopoietic cytokines produced by bone marrow endothelial cells [J]. Cytokine, 2000, 12(7):1017-1023.

(Edited by PENG Min-ning)