In vitro isolation of intestinal side population cells from newborn mice*☆

Zhang Yong-feng[1], Yang Jin[2], Wu Zheng-zhi[1], Jia Xiu-qin[1], Li Ming[1], Li Ying-hong[1]

Abstract

BACKGROUND: Current methods of stem cell separation are mainly based on their cell markers. A method for stem cells separation which is not based on cell markers developed in recent years, that is fluorescence activated cell sorting method, has been applied for stem cells and mature cells separation.

OBJECTIVE: To isolate side population cells from newborn mice small intestinal mucosa, and to investigate the feasibility of constructing the murine intestinal stem cell population by fluorescence activated cell sorting.

METHODS: Small intestine mucosa organoids of mice were isolated and dissociated into single cells. The side population cells were stained with Hoechst 33342 and propidium iodide, then sorted using fluorescence activated cell sorting. Total RNA and protein were purified from sorted fractions to detect Musashi-1 expressions by RT-PCR and Western-blotting.

RESULTS AND CONCLUSION: Single cell suspension from mouse small intestine mucosa contained a viable population of cells, which showed the side population phenotype and were sensitive to verapamil. These cells were enriched for Musashi-1 mRNA and MSI-1 protein expression. Results demonstrated that the side population fraction separated from mice intestinal mucosa is enriched for intestinal stem cells, the murine intestinal stem cell population can be successfully constructed with fluorescence activated cell sorting.

[1]Shenzhen Clinical Institute of Integrated TCM and Western Medicine, Shenzhen Second People's Hospital, Shenzhen 518035, Guangdong Province, China; [2]Hubei College of Traditional Chinese Medicine, Wuhan 430061, Hubei Province, China

Zhang Yong-feng☆, Doctor, Chief physician, Master's supervisor, Shenzhen Clinical Institute of Integrated TCM and Western Medicine, Shenzhen Second People's Hospital, Shenzhen 518035, Guangdong Province, China
szzyf2002@163.com

Supported by: a grant by Guangdong Provincial Science and Technology Ministry, No. 2008B030301285*

Received:2010-01-26
Accepted:2010-05-11
(20091126018/WL)

Zhang YF, Yang J, Wu ZZ, Jia XQ, Li M, Li YH.In vitro isolation of intestinal side population cells from newborn mice. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu.2010;14(27) : 5115-5118

[http://www.crter.cn http://en.zglckf.com]

INTRODUCTION

In recent years, the adult stem cell research has drawn attention from many scholars at home and abroad[1-3]. The *in vitro* proliferation, orientated induction and transdifferentiation potential of adult stem cells have opened up a new way for tissue repair and replacement. Intestinal stem cells play an important role in maintaining the barrier structure and integrated function of intestine, as well as repair after injury[4-5]. In this study, fluorescence activated cell sorting (FACS) system was applied to isolate side population cells from neonatal mouse intestinal mucosa, with aim to investigate the methods of *in vitro* isolation and identification of mouse intestinal stem cells, thus contributing to further understanding of influencing factors and underlying mechanism of intestinal stem cells proliferation and differentiation, which may lay a foundation for the biological characteristics study of intestinal stem cells.

MATERIALS AND METHODS

Design

In vitro cytology, observational study.

Time and setting

The experiment was implemented at the Laboratory of TCM Institute in Shenzhen Second People's Hospital between October 2008 and March 2009.

Materials

Ten newborn 5-day Kunming specimen female mice, of SPF grade, were offered by Laboratory Animal Center at Guangzhou University of Traditional Chinese Medicine (Certification No. 0019324). All experiment animals were treated according to *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of the People's Republic of China[6].

Main reagents and instruments are as follows:

Reagent and instrument	Source
Hoechst33342, propidium iodide (PI)	Sigma Corporation
Rabbit anti-MSI-1 polyclonal antibody	Chemicon
HBSS solution	Gibco
Goat anti-rabbit IgG2HRP	Beijing Zhongshan Company, China
HRP labeled GAPDH	Shanghai Kangcheng Company, China
RNA extraction kit, DAB coloration kit	Wuhan Boster Company, China
Flow cytometry	FACS420 type, BD Corporation, USA
CO_2 constant temperature incubator	MCO-15AC type, SANYO
Clean Bench	SW-CJ-1FD type, AIRTECH
Electrophoresis apparatus	DYY-6C type, China
PCR amplification machine	Biometra, UNO-Thermoblock type, USA
Gel imaging analysis system	WEALTEC, USA

Experimental methods

Isolation of newborn mouse intestinal side population cells

According to the methods described in literature [7-9], side population cells were isolated from mouse intestinal mucosa. Small intestine was sampled from SPF newborn mice of Kunming species, cutting into 0.5-cm debris along the longitudinal axis, rinsed 7–10 times with Ca-free and Mg-free 1× HBSS washing, to wash away the substances in intestine; oscillation 20 minutes at room temperature in 1× HBSS solution containing collagen type Ⅺ enzyme (300 U/mL) and neutral protease Ⅰ (100 mg/L); the reaction was terminated by using low-sugar DMEM containing 5%

volume fraction of fetal bovine serum and 2% sorbitol. After digestion, the supernatant was collected, followed by centrifugation 3 minutes at 250 r/min, to obtain organoid fragments which were resuspended in DMEM containing sorbitol after the dispersion. The above steps were repeated 5–6 times, until the size of these fragments is identical; the prepared organoid fragments were blew and shaken into a single cell suspension. The dispersed cells were resuspended in 1×HBSS at 1×10^{10}/L cells, dropping 2.5 mg/L concentration of Hoechst33342. The control group was stained with Hoechst33342, while adding 100 μ mol/L verapamil. Cells were incubated in 37 ℃ incubator 90 minutes, centrifuged and resuspended in 1×HBSS, and added with PI dye at 1 g/L, ice preservation for flow cytometer sorting.

Side population cells sorting was performed in this experiment according to the modification of the methods reported in the literature. The anterior laser and lateral laser were aroused by 100 mW argon ion laser at 488 nm wavelength. Hoechst33342 and PI were aroused by 100 mW of krypton ion laser at a range of 351–364 nm. Hoechst33342 fluorescence was measured through 440/60 am filter, while PI fluorescence was measured through 670/14 nm filter. Side population cells were defined as negative staining cells for Hoechst33342 and PI.

Identification of intestinal stem cell population

RT-PCR detection of Musashi-l mRNA expression: Total RNA of sorting cells was extracted according to instructions of RNeasy Mega total RNA extraction kit, with oligo dT15 as primers, reverse transcriptase reverse transcription for cDNA, finally the above reverse transcription products served as the cDNA template; upstream primer: 5'-CGA GCT CGA CTC CAA AAC AAT-3', downstream primer: 5'-GGC TTT CTT GCA TTC CAC CA-3' for RT-PCR amplification; conditions: denaturation 94 ℃ 2 minutes, 35 cycles of amplification 94 ℃ 15 seconds, 53 ℃ 30 seconds, 72 ℃ 30 seconds, PCR product was identified using 1% agarose electrophoresis.

Western-blot detection of Musashi-l protein expression: The cells were collected and washed with pre-cooling PBS 3 times, transferred to the EP tube. Total cellular protein was extracted using cytokeratin protein extracts (1% Triton100, 0.06 mol/L KCl, 0.5% HCl, 0.02 mmol/L Tris, pH 7.0, adding 200 mmol/L PMSF 5 mL/L) before use, followed by a centrifugation at 12 000 r/min, 4 ℃ 10 minutes. Supernatant was stored at −20 ℃. The extract total cellular protein was quantitated using Bradford method, 40 μg of protein samples obtained were processed into SDS-polyacrylamide gel electrophoresis and then transferred membrane. Taking rabbit anti-MSI-1 polyclonal antibody (1: 1 000), goat anti-rabbit IgG2HRP (1: 5 000) as the second antibody, HRP-labeled GAPDH (1: 5 000) as an internal control, Western-blot analysis was performed with the ECL luminescence substrate according to kit instructions.

Main outcome measures

RT-PCR and Western blot detection for Musashi-l mRNA and protein expression of sorting cells.

Design, enforcement and evaluation

The experiment was designed the first author, implemented by all authors, assessed by the first and sixth authors. They participated in the training of molecular biology experimental technique courses.

Statistical analysis

The second author completed statistical processing using SPSS 10.0 software.

RESULTS

Sorting side population cells by flow cytometry

Intestinal mucosa of newborn mice of Kunming species contains a specific group of cells, termed side population cells. This cell population accounts for 1.2% of total mucosa cells and is sensitive to the calcium blocker. When dyeing, the side population cells could disappear with the addition of calcium blocker verapamil.

Musashi-l expression of side population cells (stem cell group)

After 35 cycles of RT-PCR amplification, the Musashi-l mRNA bands could be seen at about 260 bp (Figure 1); Western-bolt detection showed MSI-1 protein band (Figure 2). It is indicated that the cell line expressed Musashi-1, with an asymmetric mitosis, suggesting that the cell line was stem cell lines.

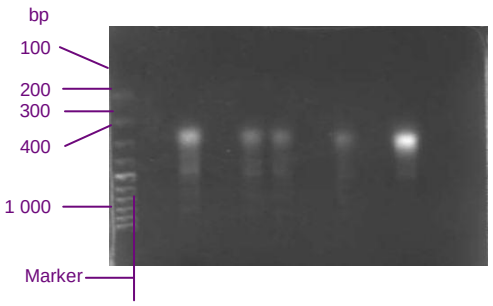

Figure 1 Electrophoresis results of RT-PCR product

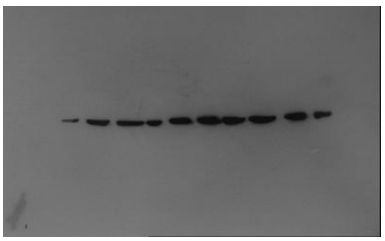

Figure 2 Western-bolt results of side population cells

DISCUSSION

There are two current methods of separating stem cells [10-12]. One is according to the stem cell specific surface markers, to use fluorescent antibody and cell surface markers, combined with flow cytometry sorting or magnetic bead sorting. The other is based on the principle of the strong vitality of cells may lead to a stronger capacity of discharging active dyes (Hoechst33342 or Rhodamine123), flow cytometry sorting for

side population cells. Many laboratories have already used this technology to separate side population cells from a variety of tissues and organs, more recent studies confirmed that side population cells are mixed cell populations enriched with stem cells and precursor cells [13].

In stem cell research, the concept of side population phenotype refers to some cells expulse fluorescent dye Hoechst 33342 which enters the cells (Hoechst 33342 is a nucleic acid binding dye and may emit blue and red fluorescence under UV excitation), showing the nucleus did not stain or low level of coloration. Further studies have shown that stem cells could express a protein, termed ABC transporter (ATP binding cassette transporter) that is ATP-dependent protein, it can discharge extracellular Hoechst 33342. The concept of side population initially develops from the separation of hematopoietic stem cells. Researchers found that hematopoietic stem cells treated with fluorescent dye Hoechst33342, were detected negative staining by flow cytometry, while stem cells isolated from other tissues also have this characteristic, by which the separated cells are known as side population cells. Numerous studies in recent years show that stem cells and some progenitor cells have the side population phenotype, and it is speculated that the side population phenotype without cell surface marker may be a sign for screening and purifying stem cell or precursor cells[14].

Side population cells have the same features and molecular characteristics of tissue-specific stem cells, and side population phenotype can be considered as functional signs of stem cells. Side population cells sorted by FACS system, can be applied to the separation and purification of stem cells in a variety of different organ systems. Due to convenient separation, economical cost and universal applicability, side population cells can be used to separate unknown surface stem cells, so as to facilitate stem cell research. Currently in the cases of absent specific cell surface markers, sorting side population cells is an effective and preferred method for isolating stem cell populations from the tissue cells [15-16].

In the study of intestinal stem cells, the key problems of the isolation, identification and *in vitro* culture are lack of a specific and reliable marker of intestinal stem cells, thus preventing the biological characteristics studies of intestinal stem cells *in vitro* and *in vivo* [17]. Recent studies show that, MSI-1 protein highly expresses in the crypt stem cells, it can be applied as an alternative specific marker of intestinal mucosa stem cells [18-20]. Since MSI-1 protein locates in the cytoplasm and is not suitable for the intestinal mucosa stem cells sorting, it also shows high expression in neural stem cells, not only in the intestinal mucosa stem cells, thus, the protein has some limitations as a specific marker, but it fits to identify the nature of sorting cells[21-22]. The results showed that side population cells isolated from neonatal mouse small intestine mucosa express MSI-1 mRNA and protein, indicating that side population cells are the enriched intestinal mucosal stem cell population.

REFERENCES

[1] Wang L, Liu J. Isolation, culture, induction, differentiation and application of hepatic stem cells. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2008;12(12):2355-2358.
[2] Wang Y, Liu H, Chen Y, et al. Progress in intestinal epithelial stem cells and colorectal cancer stem cells. Xiandai Linchuang Yixue. 2009;35(3):165-167.
[3] Wang R, Jiang JT, Yang YL, et al. Isolation and purification of tumor stem cells. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2009;13(1):161-164.
[4] Yan H. Gastrointestinal stem cells and stem cell transplantation for gastrointestinal diseases. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2007;11(20):4009-4012.
[5] Han Q, He DN, Liu YN, et al. Repair of intestinal irradiation injury by Flk-1^{-}+ MSCs and SP cells. Jichu Yixue yu Linchuang. 2008;28(6):563-569.
[6] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
[7] He DN, Qin H, Liao L, et al. Small intestinal organoid-derived SP cells contribute to repair of irradiation-induced skin injury. Stem Cells Dev. 2005;14(3):285-291.
[8] Wang ZL, Han Q, Zhao CH, et al. Characterization of intestine side population cells from fetal rats. Yixue Yanjiusheng Xuebao. 2007;20(7):693-696.
[9] Ye ZQ, Liu GL, Chen ZQ, et al. Construction of Murine Intestinal Stem Cell Population. Huazhong Keji Daxue Xuebao:Yixueban. 2006;35(6):828-830.
[10] Zhu LB, Liu ZJ, Li ZR, et al. A primary report on the culture in vitro of enteric neural stem cells in rats. Wenzhou Yixueyuan Xuebao. 2007;37(1):5-8.
[11] Li WD, Zhang XG, Chang J, et al. Isolation and culture of murine embryonic stem cells of Kunming species. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2008;12(21):4060-4064.
[12] Zhu YL, Zhong X, Zheng S. Conditionally immortalized human colorectal crypt cell line. Zhejiang Daxue Xuebao:Yixueban. 2004;33(5):379-384.
[13] Luo YQ, Wen Y, Wu CT. The advances on Isolation and culture of intestinal epithelial stem cells. Guoji Waike Zazhi. 2007;34(2):101-103.
[14] Xu YY, Zhu HY, Ai J, et al. Research Progress of SP cells. Shengming de Huaxue. 2008;28(5):536-538.
[15] Li YT, Hu WH, Dou J. SP. Research progress of side population cells. Shengwu Jishu Tongxun. 2008;19(3):430-432.
[16] He QZ, Lu HJ, Ma AG. The relationships and advances on stem cells、tumor stem cells and side population cells. Xiandai Zhongliu Yixue. 2008;16(10):1803-1806.
[17] Hao YP, Hu SM, Zhang SD, et al. Expression and significance of stem cell marker LGR5 proteins in the development and progression of human colorectal carcinoma. Shandong Daxue Xuebao: Yixueban. 2009;47(8):85-88.
[18] Asai R, Okano H, Yasugi S. Correlation between Musashi-1 and chairy-1 expression and cell proliferation activity in the developing intestine and stomach of both chicken and mouse. Dev Growth Differ. 2005;47(8):501-510.
[19] Wang YX, Wu CT. Research progress of intestinal stem cell markers. Zhongguo Putong Waike Zazhi. 2008;17(10):1013-1015.
[20] Zhao Y, Li JS, Ma CL. Identification of human gastrointestinal stem cell markers. Weichangbingxue he Ganbingxue Zazhi. 2006;15(2):199-202.
[21] Gong JF, Zhu WM, Gao X, et al. Cloning and functional analysis of the promoter region of an intestinal stem cell specific expressed gene,Musashi-1. Changnei yu Changwai Yingyang. 2006;13(5):257-260.
[22] Gu EY, Ha F, Li LF, et al. Identification method of neural stem cells. Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu. 2009;13(10):1947-1950.

新生小鼠肠侧群细胞的体外分离*☆

张永锋[1]，杨　晋[2]，吴正治[1]，贾秀琴[1]，李　明[1]，李映红[1] ([1]深圳市第二人民医院 中西医结合临床研究所，广东省深圳市　518035；[2]湖北中医药大学，湖北省武汉市　430061)

张永锋☆，男，1965年生，广东省揭西县人，汉族，2005年湖北中医药大学毕业，博士，主任医师，硕士生导师，主要从事中西医结合脾胃病的研究。

摘要

背景：目前分离干细胞的方法主要基于其细胞标志，近年来发展一种非基于细胞标志的干细胞分离方法，即利用荧光激活细胞分类法将组织中干细胞和成熟细胞分离。

目的：分离新生小鼠小肠黏膜来源的侧群细胞，探讨利用荧光活化细胞分选系统构建鼠肠干细胞群的可行性。

方法：取新生小鼠小肠全长，制作小肠黏膜的类器官片段并制备成单细胞悬液。使用Hoechst33342和碘化丙啶染色后用流式细胞仪分选侧群细胞。提取细胞总RNA和蛋白，RT-PCR及Western-blot方法分别检测其中MSI-1 mRNA及MSI-1蛋白的表达水平。

结果与结论：新生小鼠来源的小肠黏膜单细胞悬液中包含一个特定的细胞群体即侧群细胞，染色液中加入维拉帕米后，侧群细胞被阻断后消失。侧群细胞中显示有MSI-1 mRNA及蛋白的表达。提示新生小鼠的小肠黏膜侧群细胞富集小肠黏膜干细胞，荧光活化细胞分选系统可用于构建鼠肠干细胞群。

关键词：肠干细胞；侧群细胞；小鼠；Musashi-1；荧光活化细胞分选

doi:10.3969/j.issn.1673-8225.2010.27.041

中图分类号: R394.2　文献标识码: B

文章编号: 1673-8225(2010)27-05115-04

张永锋，杨晋，吴正治，贾秀琴，李明，李映红. 新生小鼠肠侧群细胞的体外分离[J]. 中国组织工程研究与临床康复, 2010,14(27): 5115-5118.

[http://www.crter.org　http://cn.zglckf.com]

(Edited by Yang Y/Wang L)

来自本文课题的更多信息--

基金资助：广东省科技厅资助项目(2008B030301285)。

利益冲突：课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

课题的创新点：近年来成体干细胞的研究是学术界关注的热点问题，对干细胞定位、生命周期、环境对其功能和基因表型的影响以及癌变等问题的研究具有深远意义，而干细胞的分离和鉴定是所有干细胞和肿瘤干细胞研究的前提，也一直是一个难题。以往文献报道小鼠(或大鼠)肠干细胞群的构建多采用胎鼠，分离用酶消化法，本文的新颖性在于新生小鼠，应用荧光活化细胞分选系统。

课题评估的“金标准”：较普遍接受的小肠上皮干细胞标志物是Lgr5，MSI-1作为肠干细胞的标志物，国内已有应用的文献报道。当前相关研究中的一个最大障碍是缺乏特异性的肠道干细胞标记物，从而制约了肠道干细胞的分离和体外培养，Musashi-1，端粒酶反转录酶，CD133和ID14等是目前研究较多的肠道干细胞的候补标记物。今后应考虑应用肠上皮干细胞标志物Lgr5，以增强说服力。

设计或课题的偏倚与不足：文章为课题前期的部分内容，系初步验证干细胞，所采用标记物MSI-1作为小肠上皮干细胞的标志物尚未得到共识，验证干细胞的方法为间接方法，随着研究的深入，如需证实干细胞，考虑增加直接鉴定方法(体内或体外证实其多向分化特性)。

提供临床借鉴的价值：肠道干细胞在维持肠道结构与功能的完整以及损伤后的修复起关键作用。肠道疾病的病理改变，导致肠道黏膜，甚至黏膜下肌层的结构和功能受到破坏，通过干细胞和组织工程技术定向诱导肠道干细胞使其分化为特定的细胞以替代功能障碍的细胞或组织缺损，这可能是治疗肠道疾病的根本方法。

脐带间充质干细胞的培养与分化：本刊中文部②

5　脐带间充质干细胞的体外分离及生物学特性观察

田　新(广西医科大学第四附属医院儿科，广西壮族自治区柳州市　545005)

推荐理由：脐带组织依赖于母体免疫系统的保护，且胚胎自身免疫系统相对不发育、MHC表达低下，故来源于脐带的间充质干细胞的生物学特性已成为目前研究热点。

实验采用去除血管后获取脐带组织细胞的方法可获得贴壁生长的细胞，脐带组织存在具有分化能力的间充质干细胞，并可在体外进行培养扩增形成集落细胞传代，传代细胞表达基质细胞表面抗原，能够向成骨细胞、成脂肪细胞方向分化。见2009年32期6340页。

6　体外诱导人脐带间充质干细胞向胰岛β样细胞的分化

于洪宇(辽宁医学院护理学院，辽宁省锦州市　121001)

7　脐带间充质干细胞对脐血CD34+细胞体外扩增的影响

袁晓莉(中国医学科学院中国协和医科大学血液学研究所、实验血液学国家重点实验室，天津市　300020；　河南省人民医院，河南省郑州市　450003)

8　脐带沃顿胶间充质干细胞的分离培养及其诱导分化

蒋洁(中南大学湘雅基础医学院组织学与胚胎学系，湖南省长沙市　410013；怀化医学高等专科学校组织学与胚胎学教研室，湖南省怀化市　418000)

9　人脐带间充质干细胞分离培养及向脂肪与成骨细胞的分化

何绍清(暨南大学生物医药研发基地，广东省广州市　510632；广州(暨南)-香港细胞工程联合实验室，广东省广州市　510632)

10　肝细胞生长因子与成纤维生长因子联合诱导人脐带间充质干细胞向肝样细胞的分化

朱华民(暨南大学医学院血液病研究所，广东省广州市　510632)

详见：http://www.crter.org/sites/MainSite/DetailD.aspx?StructID=12171