Feeder-free growth of human embryonic stem cells supported by basic fibroblast growth factor*☆

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Abstract

BACKGROUND: Human embryonic stem cells (hESCs) are pluripotent cells which may differentiate into tissues of all three germ layers. Such research as the feeder-free growth of hESCs is few in China. Fibroblast growth factor (FGF) is a major factor to maintain the undifferentiated state of hESCs.
OBJECTIVE: To evaluate the ability of FGF at different concentrations in maintaining the undifferentiated state and pluripotency of hESC lines in the long-term culture.
METHODS: Two cell lines of hES-8 and hES-18 were cultured with mouse embryonic fibroblast condition medium for 3 passages and then transferred into mouse embryonic fibroblast condition medium containing different concentrations of FGF: 100, 160, 250 μg/L for 8 passages. The hESCs were removed from the petri dish, cell clusters were digested with collagenase IV and gathered. Cell differentiation and pluripotency were observed. The eighth generation of the hESCs were collected and incubated into severe combined immunodeficiency mice, so as to observe teratoma formation. Morphologies of the cells were evaluated. Alkaline phosphatase staining, surface labeling immunocytochemical analysis and RT-PCR assay method were utilized to determine the OCT-4 expression and tumorigenesis *in vivo*.
RESULTS AND CONCLUSION: Cultured in mouse embryonic fibroblast condition medium containing 160 and 250 μg/L FGF, two cell lines of hESC could maintain undifferentiated state: Clones were round with a high ratio of nucleus to cytoplasm. Large areas in the center of clones were undifferentiated cells, while surrounding the clones were differentiated cells; Strong positive expression for alkaline phosphatase staining was observed; Two cell lines showed high levels of OCT-4 transcription factor protein; The surface markers SSEA-4, TRA-1-60, TRA-1-81 were all positive on both two lines; The hESC clusters could form embryoid body *in vivo* 10 days later; 3 germ layers of teratomas were also obtained after implanted into severe combined immunodeficiency mice. Mouse embryonic fibroblast condition medium containing 100 μg/L FGF was not sufficient to maintain the long-term proliferation of hESCs, and most of the cells differentiated and died after 4 passages. Alone with concentration 160 μg/LbFGF or more could maintain two hESC lines undifferentiated stably *in vitro*, has no influence on the differentiation and totipotency of two cell lines.

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INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent cells which can differentiate into tissues of all 3 germ layers[1-3] and have been derived from the inner cell mass of human embryos[4]. Maintenance of self-renewal and pluripotency may be the most important factor for the hESCs culture. Basic fibroblast growth factor (bFGF) has been widely used to support hESCs either in feeder-layers or in feeder-free culture systems[4-5]. Recently some researchers have reported that bFGF could sustain hESCs undifferentiated in the long-term culture[6-7]. Here we evaluated the ability of bFGF in the maintenances of undifferentiated state and pluripotency of two hESC lines.

MATERIALS AND METHODS

Materials

This study was carried out in the Reproductive Medical Center, Tongji Hospital, Huazhong University of Science and Technology between May 2007 and June 2008. Two Chinese cell lines hES-8, 46XX and hES-18, 46XY were derived and identified by Dr. Chen Hong of this research group[8].

Methods

Cell culture

Two cell lines of hES-8 46XX and hES-18 46XY were cultured on mouse embryonic fibroblasts conditional medium for 3 passages and then transferred to mouse embryonic fibroblasts conditional medium containing 100, 160, 250 μg/L bFGF (FM-100, FM-160, FM-250) for another 8 passages[9]. Embryonic stem cell medium for feeder-layer culture system consisted of 80% knockout Dulbecco Modified Eagle Medium (DMEM, Gibco, USA) and 20% Knockout serum replacement (Gibco, USA) supplemented with 2 mM L-glutamine (Gibco, USA), 1% nonessential amino acids, 0.1 mM β-mercaptoethanol (Sigma, USA), 100 U/ml penicillin-streptomycin (Hyclone, USA) and 4 ng/ml human bFGF (Peprotech, USA). Mouse embryonic fibroblast feeder-layer was cultured with embryonic stem cell medium for 24 hours and then supernates were collected as condition medium. The bFGF at high concentrations were added to embryonic stem cell medium to make FM.

Immunocytochemical analysis

Cells were fixed with dehydrated alcohol for 10 minutes at room temperature. After incubation with anti-SSEA4, anti-TRA-1-60 and TRA-1-81 (all from Chemicon, USA), the cells were washed with PBS and incubated with biotinglated secondary antibodies IgG or IgM and then with streptavidin peroxidase regent (Zhongshan Golden Bridge Biotechnology, China). Diaminobenzidine (Zhongshan Golden Bridge Biotechnology, China) was used to localize the secondary antibodies. The surface labeling SSEA4, TRA-1-60 and TRA-1-81 immunocytochemical analysis were performed.

Alkaline phosphatase activity

The cells were fixed with dehydrated alcohol for 10 minutes at room temperature. The alkaline phosphatase assay was performed with AP-Red Substrate Kit (Zhongshan Golden Bridge Biotechnology, China).

Expression of OCT4

Total RNA was extracted from cells at 8 passage by using the RNA Simple Total RNA Kit (Tiangen Biotech, China) according to the manufacturer's recommended protocols. First strand cDNA synthesis were carried out using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas Lithuania). The primers and PCR reaction was performed as previously described[8]. The PCR products were analyzed on 2% agarose gels. The OCT-4 expression was determined using RT-PCR method.

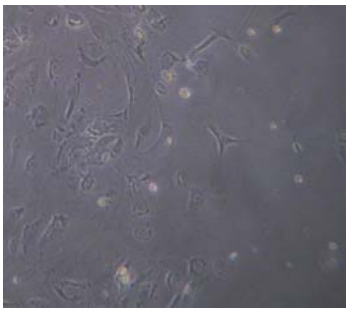
In vitro and in vivo differentiation

The hESCs were removed from the petri dishes and digested into small clusters using 1 mg/ml Collagenase IV. Such cell clusters were then cultured in 35-mm plastic dishes (BD, USA) with embryonic stem cell medium without bFGF. To examine the capacity to generate teratomas of two cell lines cultured in medium, cells after 8 passages were collected and implanted into severe combined immunodeficiency mice as described previously[8] to observe teratoma formation.

RESULTS

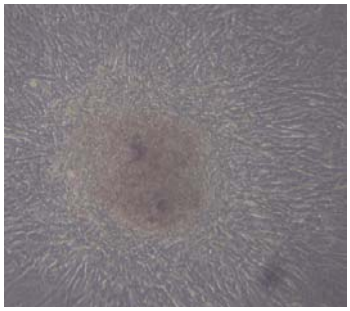
Growth of two cell lines

Two cell lines hES-8 and hES-18 could maintain all their characteristics in feeder-free culture with FM-160 and FM-250. FM-100 with 100 ng/ml bFGF was not sufficient to maintain two cell lines undifferentiated in the long-term culture and after 4 passages most of the cells differentiated and died (Figure 1). For FM-160 and FM-250, the growths of cells were similar as morphology was concerned: clones were round with a high ratio of nucleus to cytoplasm. Large area in the center of clones were undifferentiated cells, while surrounding clones were differentiated cells. Two cells lines were passaged once every 10 days and differentiation rates of two lines were about 20%-25% by morphology on FM-160 and FM-250 (Figure 2).



FM: mouse embryonic fibroblast condition medium containing fibroblast growth factor (100 μg/L)

Figure 1 Morphology of human embryonic stem cells cultured with FM100, hES-18, most of the cells differentiated and died (×100)



FM: mouse embryonic fibroblast condition medium containing fibroblast growth factor (160 μg/L)

Figure 2 Morphology of human embryonic stem cells cultured with FM160, hES-18 (×100)

Identification of hESCs

Two cell lines cultured with FM-160 and FM-250 showed high levels of alkaline phosphatase activity (Figure 3) and transcription factor OCT-4 (Figure 4).

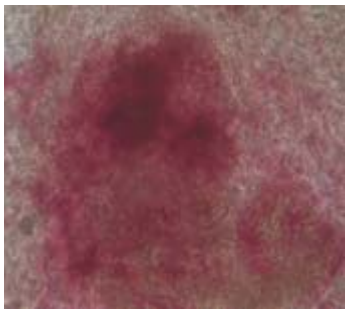
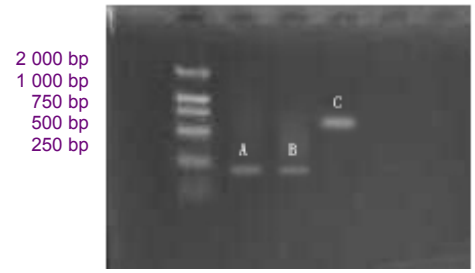


Figure 3 Detection of alkaline phosphatase in human embryonic stem cells, FM160, hES-8 (Inverted phase contrast microscope, ×100)



A: hES-8; B: hES-18; C: Marker

Figure 4 RT-PCR of the expression of OCT-4 on two cell lines in the culture system

The surface markers SSEA-4, TRA-1-60, TRA-1-81 were positive on both two lines (Figure 5). When the clusters hESCs were cultured for 10days, they formed embryoid body (Figure 6). After cultured on FM-160 and FM-250, two hESC lines were implanted into severe combined immunodeficiency mice and then form teratomas which included tissues of 3 germ layers (Figure 7).

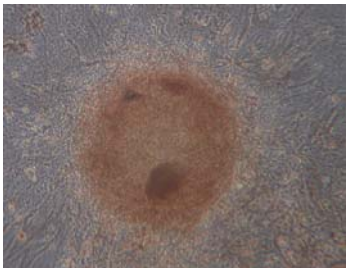


Figure 5 Detection of surface makers SSEA-4 of undifferentiated human embryonic stem cells, FM160, hES-18 (Inverted phase contrast microscope, ×100)

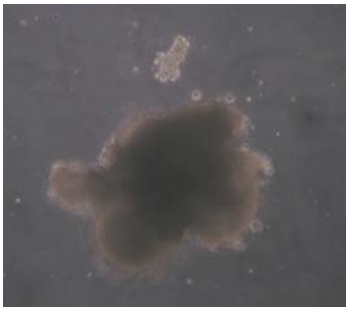


Figure 6 The formation of embryoid bodies, FM250, hES-18 (Inverted phase contrast microscope, ×100)

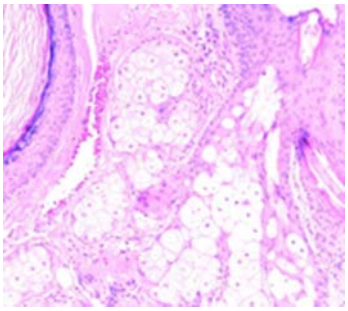


Figure 7 Teratoma on severe combined immunodeficiency mice, FM160, hES-8 (Hematoxylin-eosin, light microscope, ×100)

DISCUSSION

Traditionally, hESCs have been cultured on mouse embryonic fibroblasts-feeder layer or using human cell lines as feeder cells[10-12]. The use of feeder cells limits the research works because the combination of hESCs and feeder cells affected the data we got. Feeder-free propagation of hESCs was first reported by using matrigel and mouse embryonic fibroblasts conditional medium[9]. Mouse embryonic fibroblasts conditional medium was obtained from mouse embryonic fibroblasts-feeder layer, which components was not defined but still contained animal components. Then researchers have tired to use several cytokines such as bFGF, transforming growth factor β[13], noggin[14], Activin[15], wnts[16] and so on, to replace conditional medium. Usually two or more cytokines are used together to maintain the non-differentiated growth unlike LIF on mouse ESC which is sufficient independently[17]. Among the cytokines, bFGF is widely used by many research groups. The precise mechanism of action for bFGF in maintaining the self-renewal of hESCs remains unclear. The data from many research groups confirmed the role of bFGF in supporting hESCs growth. BFGF can inhibit maturation of oligodendrocyte precursors[18] and may interact with its receptors to block differentiation of hESCs[19]. Previous researches indicated that hESCs derived from different origins showed different characteristics[20-30]. We found the rate of differentiation was higher and proliferation time was longer when used conditional medium system. Here 10 days for proliferation and 20%–25% differentiation rate confirmed again. It should be noticed that the concentration was higher too (compared with 100 ng/ml reported abroad). Maybe hESCs of Chinese origin really have different growth characteristics to a certain degree.

It is the first time to use bFGF for supporting two Chinese hESCs lines undifferentiated growth independently. This culture system may limit the animal components and also simplify our research works.

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成纤维细胞生长因子支持人胚胎干细胞在无饲养层中的生长*☆

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摘要

背景：人胚胎干细胞是一种全能型细胞，可以分化为 3 个胚层的组织，目前国内对其无饲养层生长的研究较少。成纤维细胞生长因子是维持胚胎干细胞不分化状态的重要因子。

目的：探讨长期培养过程中不同质量浓度成纤维细胞生长因子对人胚胎干细胞未分化状态和全能性维持的影响。

方法：两株人胚胎干细胞在鼠胚胎成纤维细胞条件培养基中培养 3 代，分别转移到含 100，160，250 μg/L 成纤维细胞生长因子的鼠胚胎成纤维细胞条件培养基中培养 8 代。从培养皿中移出胚胎干细胞，用 IV 胶原酶消化聚集成团的细胞，观察细胞分化状态和全能性情况。收集传 8 代后的胚胎干细胞，种植于 SCID 小鼠体内。对所得细胞做形态学评估，并进行碱性磷酸酶染色、表面标记免疫组化检测、RT-PCR 检测 OCT-4 的表达、体内致瘤情况。

结果与结论：在含 160，250 μg/L 成纤维细胞生长因子的鼠胚胎成纤维细胞条件培养基中，两株人胚胎干细胞可以保持原有性状，即细胞克隆呈圆形，核质比较高，中间大片区域为未分化细胞，周围为分化细胞；呈碱性磷酸酶强阳性表达；表达 OCT-4 转录因子蛋白；细胞表面标志 SSEA-4，TRA-1-60，TRA-1-81 均呈阳性表达；聚集成团的胚胎干细胞培养 10 d 后形成拟胚体；种植于 SCID 小鼠体内可得含 3 个胚层组织的畸胎瘤。含 100 μg/L 成纤维细胞生长因子的鼠胚胎成纤维细胞条件培养基不足以维持人胚胎干细胞的长期增殖，4 代以后大部分细胞分化死亡。提示成纤维细胞生长因子质量浓度达 160 μg/L 以上时，可以单独支持人胚胎干细胞的体外稳定增殖，且不影响细胞分化状态和全能性。

关键词：人胚胎干细胞；成纤维细胞生长因子；无饲养层；培养
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