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Establishment of Feeder Layer-Free and Serum-Free Isolation and Culture System for Mouse Embryonic Stem Cells

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Abstract: The aim of this study was to establish a feeder layer-free and serum-free isolation and culture system for Kunming mouse Embryonic Stem Cells (ESCs). Mouse ESCs were isolated and cultured in Mouse Embryonic Fibroblasts (MEFs) feeder layer and Fetal Bovine Serum (FBS) culture system or feeder layer-free and serum-free culture system respectively and their primary colonies formation rates, maximum passage number and morphology and growth characters were compared. The undifferentiated status were identified by expression of Alkaline Phosphatase (AKP), Octamer-binding transcription factor 4 (Oct-4) and Stage-Specific Embryonic Antigen 1 (SSEA-1). The results indicated that there were no significant difference in the primary colonies formation rates ($p<0.05$) and no obvious difference in maximum passage number of ESCs between basal ESCs medium with MEFs feeder layer and FBS treated groups and modified ESCs medium without MEFs feeder layer and serum treated groups, respectively. There were some differences in morphology and growth characters of ESCs between those two kinds of culture systems. ESCs cultured in feeder layer-free and serum-free culture system were positive for AKP activity and the immunocytochemical staining studies revealed positive reaction to Anti-Oct-4 monoclonal antibody and Anti-SSEA-1 monoclonal antibody, respectively. Therefore, mouse ESCs isolated in this study can grow in feeder layer-free and serum-free culture system and maintain their self-renewal and undifferentiated state.

Key words: Kunming mouse, embryonic stem cells, mouse embryonic fibroblasts, feeder layer-free and serum-free culture system, keep undifferentiated state

INTRODUCTION

Embryonic Stem Cells (ESCs) originate from the epiblast of the Inner Cell Mass (ICM) of the preimplantation blastocyst or Primordial Germ Cells (PGCs), these pluripotent cells have the ability to self-renew and be maintained in an undifferentiated state indefinitely while maintaining the potential to be induced to differentiate into derivatives of all three embryonic germ layers under *in vitro* and *in vivo* conditions, they are potentially valuable for the basic research and clinical application including researches on development of early embryo, producing transgenic animals, screening drugs and toxins, establishing animal models, cell transplantation, gene therapy, etc. In some species, ESCs had been isolated under different conditions. ESCs were first isolated in 1981 from mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981) and subsequently from porcine primordial germ cells (Shim *et al*., 1997), bovine primordial germ cells (Cherny and Merei, 1994), preimplantation rabbit embryos (Graves and Moreadith, 1993), ovine and caprine preimplantation embryos (Meinecke-Tillmann and Meinecke, 1996), rhesus monkey blastocysts (Thomson *et al*., 1995) and human blastocysts (Bongso *et al*., 1994; Thomson *et al*., 1998). Tight conditions of culture *in vitro* must be taken to ensure that ESCs can be proliferated indefinitely and be maintained in an undifferentiated state. The traditional method is that ESCs were cultured on the Mouse Embryonic Fibroblasts (MEFs) feeder layer. But preparation of MEFs feeder layer is complex, experimental error possibly resulted from the different batches of MEFs, the compositions of secretion from MEFs feeder layer were difficult to be identified and that was not conducive to study the molecule mechanism of self-renewal and induced differentiation of ESCs, ESCs were possibly contaminated with heterogeneous gene and protein, animal pathogens, mycoplasma, exogenous serum, etc. In recent years, scientists had made great efforts to optimize the culture conditions for ESCs and

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achieved considerable progresses forward. The researches about ESCs cultured under feeder layer-free and serum-free conditions has been a hotspot in medical field.

In this study, the colonies formation rates, maximum passage number, morphology and growth characters and undifferentiated status of ESCs which were isolated and cultured in MEFs feeder layer and Fetal Bovine Serum (FBS) culture system or feeder layer-free and serum-free culture system were compared in order to establish a feeder layer-free and serum-free isolation and culture system for ESCs and lay the foundation for the further researches on induced differentiation and gene-transfection of ESCs.

MATERIALS AND METHODS

All Kunming mice at 6-8 weeks age were obtained from Chongqing Academy of Chinese Material Medica. Unless otherwise mentioned, all reagents used were obtained from Sigma.

Superovulation of mouse: Female Kunming mice were superovulated with 10 IU Pregnant Mare Serum Gonadotropin (PMSG, Ningbo Second Hormone Factory) followed at 48 h by 10 IU human Chorionic Gonadotropin (hCG, Ningbo Second Hormone Factory), joined with male Kunming mice and examined for a vaginal plug at 9:00 tomorrow morning. Those with a vaginal plug were isolated.

Preparation of MEFs feeder layer: Mouse embryos at 13.5 days post coitum (dpc) were aseptically dissected from pregnant mice, the embryos were placed in a 10 cm sterile glass petri dish containing sterile Ca^{2+} free and Mg^{2+} free Phosphate-Buffered Saline (D-PBS). The embryo's limbs, internal organs, the brain or upper part of the head containing the brain were removed. The remain tissues were minced into very small pieces with a sterile surgical scissors. The minced embryos were placed into sterile penicillin bottle containing 0.25% trypsin and 0.4% Ethylenediamine Tetraacetic Acid (EDTA) in D-PBS and incubated at 37°C for 30 min before being inactived by 10% FBS (NQBB) in DMEM (Thermo) solution and centrifuged at 1000 rpm for 5 min at least twice. Cell culture flasks contained 2 mL MEFs suspension were placed into incubator at 37°C, 5% CO_2. The medium (DMEM supplemented with 15% FBS, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) was changed the next day and the cells were allowed to grow until the bottom of cell culture flasks were confluent.

Third-passage MEFs were used to prepare the feeder layer. Third-passage MEFs feeders were mitotically inactivately by being treated with 10 μg mL^{-1} mitomycin C for 2-3 h then cells were washed with D-PBS, trypsinized and centrifugated. The supernatant was removed and the pellet was resuspended with fresh medium for MEFs culture. Cells were counted and seeded (2×10^5 cells mL^{-1}) into cell culture dishes.

Collection of mouse blastocyst: Time-mated female mice were killed by cervical dislocation at 3.5 dpc and their uteri were immediately transferred into M_2 medium in a 35 mm glass culture dish. Remnants of fat were removed, the uteri were transferred into 2 mL of fresh preheated M_2 medium and the blastocysts were flushed out from each uterine horn with about 0.2 mL of M_2 medium using a 1 mL syringe with a 26-gauge hypodermic needle.

Morphologically normal blastocysts were collected and washed through several drops of fresh M_2 medium to rinse off the debris. Then, those blastocysts were randomly transferred to microdrops of DMEM supplemented with 15% FBS on MEFs feeder layer or DMEM supplemented with 15% Knockout Serum Replacement (SR) in glass culture dish which precoated with 10 mg L^{-1} laminin.

Isolation and culture of ICM: ICM-derived clumps were dislodged from the underlying sheet of trophoblast cells using a finely drawn Pasteur pipette. ICM clumps were washed twice with D-PBS then those cells were transfered into the microdrops of D-PBS supplemented with 0.125% trypsin and 0.2% EDTA. The microdrops were incubated at 37°C for 3-4 min. ICM clumps were blew gently and repeatedly and disaggregated into smaller cellular aggregates which were transfered into the fresh microdrops of basal ESCs medium on the MEFs feeder layer or modified ESCs medium without feeder layer and serum in glass culture dish which precoated with 10 mg L^{-1} laminin after those smaller cellular aggregates had been washed twice with the respective culture medium.

The basal ESCs medium was composed of DMEM supplemented with 15% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin and the modified ESCs medium was composed of DMEM supplemented with 15% Knockout SR, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 10 ng mL^{-1} Leukemia Inhibitory Factor (LIF), 20 ng mL^{-1} basic Fibroblast Growth Factor (bFGF), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The medium was changed every 2 days.

Isolation, culture and passage of ESCs: Compact cell colonies resembling ESCs colony morphology could be detected after culture *in vitro*. These cells could be subsequently expanded and trypsinized according to needs. All cells in a dish were trypsinized with 0.125% trypsin and 0.2% EDTA when the cell clumps were lifted off and disaggregated, the resulting cells were washed with basal ESCs medium or modified ESCs medium and seeded onto new dishs in the ratio of 1:3 to 1:7, ESCs were allowed to grow with medium changes every 2nd day. Usually, an additional trypsinization step was needed when the culture reaches about 70% confluence for the passage of ESCs.

Observation of morphology and growth characters of ESCs: Morphology and growth characters of ESCs were observed by inverted microscope (OLYMPUS, Japan Olympus Optical Co., Ltd.).

Alkaline Phosphatase (AKP) staining: AKP was stained by using AKP detection kit (ZSGB-BIO, K105215A) following the manufacture's instructions.

Immunocytochemistry: Octamer-binding transcription factor 4 (Oct-4) and Stage-Specific Embryonic Antigen 1 (SSEA-1) immunocytochemistry were performed with SP-9002 Histostain™-Plus kits (ZSGB-BIO, 720219A). Cells were washed with D-PBS, fixed in 3% H_2O_2 for 5-10 min at Room Temperature (RT), washed with D-PBS, exposed to 5% normal goat serum in PBS for 10-15 min and incubated with anti-Oct-4 monoclonal antibody (Millipore, NG1752516) with 1:100 dilutions or anti-SSEA-1 monoclonal antibody (Millipore, LV1751783) with 1:50 dilutions overnight at 4°C. After being washed with D-PBS, cells were incubated with biotin-labeled goat Anti-mouse IgG for 10-15 min, washed and incubated with streptavidin-peroxidase for 10-15 min, washed and stained by using Diaminobenzidine (DAB) kit (ZSGB-BIO, K113324A30) and visualized under a inverted microscope. The differentiated cells appears colorless and undifferentiated ESCs colonies appears brown or reddish brown.

Statistical analysis: The experiments were performed for 3 times. The primary colonies formation rates were reported as mean±SD. Statistical analysis was performed by using SPSS (Ver. 13.0, SPSS Inc., Chicago, IL, USA). Data was analyzed by Independent-Samples t-test.

RESULTS AND DISCUSSION

Effects of different culture systems on isolation and culture of ESCs: As shown in Table 1, there was no significantly difference in the primary colonies formation rates (45.39±4.42% vs. 36.48±7.33%, $p>0.05$) and no obvious difference in maximum passage number (12 vs. 11) of ESCs between basal ESCs medium with MEFs feeder layer and FBS treated groups and modified ESCs medium without MEFs feeder layer and serum treated groups, respectively.

Morphology and growth characters of ESCs: After 1-2 days of culture on the MEFs feeder layer, the embryos hatched from the zone pellucida and attached to the surface of the culture dish with spreading of the trophoblast cells. Shortly after embryo attachment, ICM became readily distinguishable and could be seen to grow rapidly over the next days. After 1-2 days, the ICM was considerably enlarged (Fig. 1a). After trypsinized ESCs were cultured on MEFs feeder layer for 1 day, small ESCs colonies were observed. The 2 days after plating, those cells aggregated to form bigger colonies with clear edge and smooth outlines, presented the insular prominence. Differentiated ESCs were not been observed on the edge of colonies. ESCs were small and round and had a relatively bigger nucleus compared with little cytoplasm there was a obscure distinction between two edges of neighbor cells within a colony (Fig. 1b).

When the ESCs cultured in MEFs feeder layer-free and serum-free culture system, the growth of those ESCs was more slowly compared with cultured on MEFs feeder layer and the doubling time was significantly prolonged but those ESCs had typical mouse ESCs morphology. After cultured for 2-3 days, smaller colonies were observed and they could not formed colonies with glossy surface and presented the insular prominence but most formed irregular colonies with growth characteristics of flat and adherent cells, a few of ESCs were scattered in the culture dish. Single ESCs with homogeneous growing status and arranged compactly (Fig. 1c).

AKP staining and immunocytochemistry: ESCs cultured in the MEFs feeder layer and FBS culture system or in MEFs feeder-free and serum-free culture system were positive for AKP staining (Fig. 2a and d) and the immunocytochemical staining of ESCs cultured in those two kinds of culture systems revealed positive reaction to

Table 1: Effects of different culture systems on isolation and culture of mouse ESCs

Culture systems	The total No. of blastocysts	The primary colonies formation rates (%)	Maximum passage number
Basal ESCs medium with MEFs feeder layer and FBS	110	45.39±4.42[a]	12
Modified ESCs medium without MEFs feeder layer and serum	116	36.48±7.33[a]	11

Within the same column, same letter represents no significant difference ($p>0.05$)

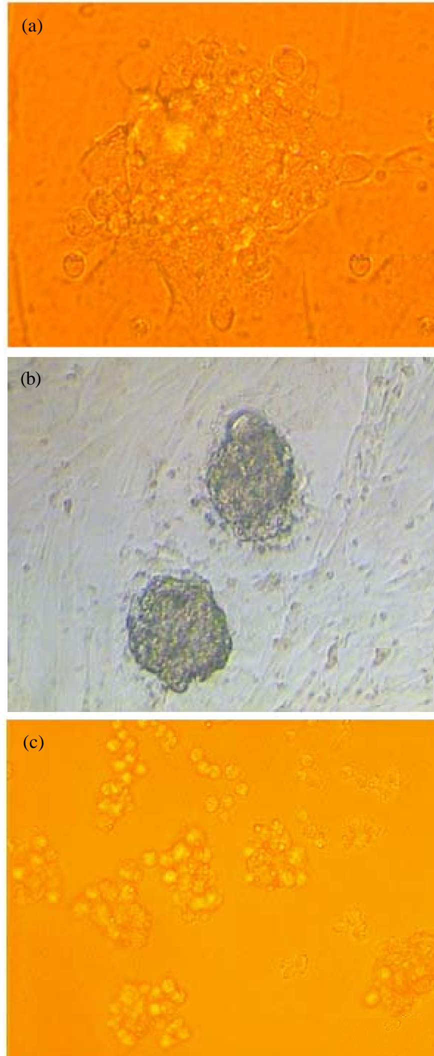

Fig. 1: Morphology and growth characters of ESCs; a) ICM from hatched blastocyst cultured on the MEFs feeder layer (x400); b) mouse ESCs in MEFs feeder layer and FBS culture system (x400) and c) mouse ESCs in feeder layer-free and serum-free culture system (x400)

Anti-Oct-4 monoclonal antibody (Fig. 2b and e) and Anti-SSEA-1 monoclonal antibody (Fig. 2c and f), respectively.

To date, the most commonly used feeder layer cells are MEFs, much cell factors and metabolic products from MEFs can support the proliferation and maintain the undifferentiated and multipotency status of ESCs.

However, MEFs have several serious limitations as feeder layer cells. First, they have a limited life span in culture as they can only be passaged 7-9 times before senesce. Second, proliferation of ESCs would be affected by remnant mitomycine C. Third, MEFs can not be used to screening of ESCs which transfected with exogenous gene owing to MEFs can not be resistance to the drugs for screening. Forth, the use of MEFs introduces the risk of contaminating ESCs with exogenous cells, genes, proteins or pathogens, etc. The medium supplemented with serum also has the risk of contaminating ESCs with exogenous proteins or pathogens, etc.

Human Embryonic Stem Cells (hESCs) were typically cultured with animal-derived serum replacements on mouse feeder layers both of these were sources of the nonhuman sialic acid Neu5Gc, against which many humans had circulating antibodies and resulted in immunoreaction (Martin *et al*., 2005) which greatly limitted MEFs were used for isolation and culture of hESCs and the possibility of those hESCs were used for treatments of clinical diseases.

Therefore, much efforts had gone into the studies of the culture systems using human different kinds of cells as feeder layer cells allowed production of hESCs such as fetal muscle cells, fetal skin cells and adult fallopian tubal epithelial cells (Richards *et al*., 2002), foreskin fibroblasts (Hovatta *et al*., 2003), adult marrow cells (Cheng *et al*., 2003), uterine endometrium cells (Lee *et al*., 2005), adult breast parenchymal cells and embryonic fibroblasts (Lee *et al*., 2004) and placental fibroblasts (Genbacev *et al*., 2005).

But not all human feeder layer cells could prolong undifferentiated growth of hESCs (Richards *et al*., 2003). Furthermore, the risks of cross-transfer of pathogens from xenogeneic or allogeneic feeder layer cells or cell by-products will limit medical applications of hESCs. Therefore, researchers attempted to use the feeder layer cells differentiated from the hESCs, e.g., fibroblast-like cells derived from the spontaneous differentiation of the hES-NCL1 and commercially available hESCs (H1 line) could be used as an autogeneic feeder system that efficiently supported the growth and maintenance of pluripotency of both autogeneic and allogeneic undifferentiated hESCs (Stojkovic *et al*., 2005). Fibroblast-like cells differentiated from the Miz-hES6 hESCs line were used to support the *in vitro* growth of three hESCs lines, all three hESCs lines were maintained in an undifferentiated state (Yoo *et al*., 2005). But the number of fibroblast cells differentiated from hESCs was limited which greatly limited the medical application of this autologous culture system for hESCs. Lately, a hESCs line (SNUhES3) was cultured on human placenta-derived mesenchymal stem cell feeder layer, the maintenance of undifferentiated state of SNUhES3 was demonstrated by

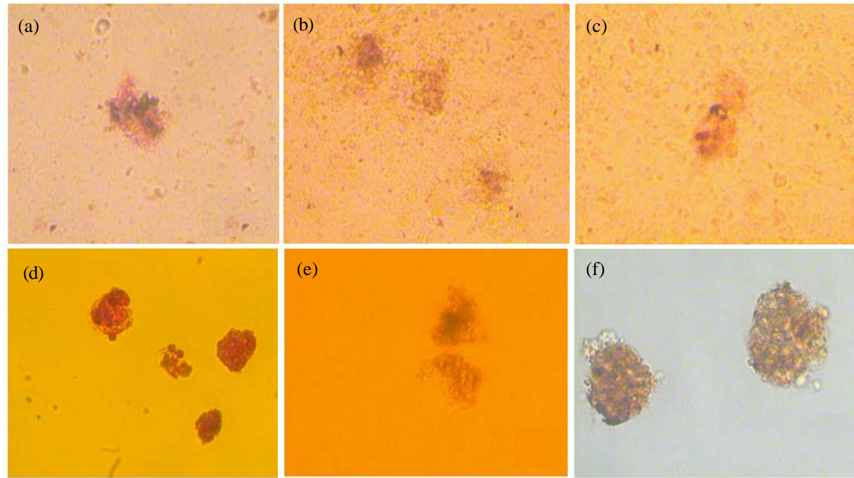

Fig. 2: AKP staining and immunocytochemical staining of ESCs; a) ESCs cultured in MEFs feeder layer and FBS culture system were positive for AKP staining (x200); b) ESCs cultured in MEFs feeder layer and FBS culture system were positive for Oct-4 immunocytochemical staining (x200); c) ESCs cultured in MEFs feeder layer and FBS culture system were positive for SSEA-1 immunocytochemical staining (x200); d) ESCs cultured in feeder layer-free and serum-free culture system were positive for AKP staining (x400); e) ESCs cultured in feeder layer-free and serum-free culture system were positive for Oct-4 immunocytochemical staining (x400) and f) ESCs cultured in feeder layer-free and serum-free culture system were positive for SSEA-1 immunocytochemical staining (x400)

the expression of SSEA-4, TRA-1-81, TRA-1-60 and Oct-4 after 50 weeks (Kim *et al*., 2007). Although, the use of human feeder layer from hESCs reduced some biologic risks, there were still some problems in using those culture systems such as preparation of feeder layer was complex, not all batches of human feeder layer could support the culture of undifferentiated hESCs equally well, many factors secreted from human feeder layer had not yet been fully identified and that resulted in the difficulties to analysis the molecular biology mechanism in supporting the proliferation and maintaining the undifferentiated states of hESCs. Therefore, feeder-free and serum-free culture system of hESCs are concerned widely.

The usually methods of feeder layer-free culture system of ESCs were those cells cultured in condition media supplemented with factors supporting proliferation and inhibiting differentiation of ESCs or condition media prepared with cells which could secrete those factors. It had been reported that condition media for feeder layer-free culture of hESCs were made with some kinds of cells such as PSA-1 embryonic carcinoma cells (Martin, 1981), Buffalo rat liver cells (Huang *et al*., 2000), heart tissues of a 2-3 weeks postnatal rat (Han *et al*., 1997), chicken liver cell lines (Yang and Petitte, 1994), MEFs (Xu *et al*., 2001), human fibroblasts (Bigdeli *et al*., 2008), human bladder carcinoma cell line 5637 (Pease *et al*., 1990), T3-cell line (Strelchenko and Niemann, 1993) and transgenic human fetal liver stromal cells (He *et al*., 2010). The exogenous cell factors supplemented in feeder layer-free and serum-free culture media generally were included two types, factors inhibiting the differentiation of ESCs, e.g., LIF and Transforming Growth Factor β1 (TGF-β1) and growth factors supporting the proliferation of ESCs, e.g., bFGF, Epidermal Growth Factor (EGF). Murine ESCs could be maintained as a pluripotent, self-renewing population by LIF/STAT3-dependent signaling (Cartwright *et al*., 2005). In the presence of 10 ng mL^{-1} recombinant LIF the totipotent D3 ESCs were maintained for over 2 months as undifferentiated cells in the absence of any feeder layer cells (Shirley and Williams, 1990). Basic FGF supported undifferentiated growth of hESCs, the highest concentration (8 ng mL^{-1}) given the best result (Koivisto *et al*., 2004). Although, some researchers had been taken to explore the mechanism of FGF in maintaining the undifferentiation state of ESCs, e.g., withdrawal of FGF2 from the media led to acquisition of typical differentiated characteristics in hESCs. FGF2 maintained hESCs self-renewal by supporting stable expression of extracellular matrix molecules through activation of the PI3K/Akt/PKB pathway (Kim *et al*., 2005) and basic FGF activated ERK and induced c-fos in hESCs line MizhES1 (Kang *et al*., 2005), the real mechanism must be studied further.

Therefore, some researchers attempted to explore the useful chemical composition of ESCs media and establish the feeder layer-free and serum-free ESCs culture system.

Several possible combinations of growth factors including TGF-β1, LIF and bFGF were tested for their ability to support the maintenance of undifferentiated hESCs, the results indicated that a serum-free medium supplemented with TGF-β1 and bFGF or TGF-β1, LIF and bFGF were found to be the most suitable for the culture of undifferentiated hESCs (Amit *et al*., 2004). Rhesus monkey ESCs could be cultured through at least 22 passages on laminin in feeder layer-free and serum-free medium supplemented with SR, bFGF and TGF-β1. The hESCs retained their undifferentiated state when cultured in using the Vitronectin (VN), insulin-like growth factor-I and insulin-like growth factor binding protein-3 combinations as a serum-free medium for up to at least 10 passages (Richards *et al*., 2008). EGF receptor inhibitors SU5402 supplemented in ESCs medium supports ESCs propagation (Ying *et al*., 2008). EGF partially stimulates proliferation of mouse ESCs via., PLC/PKC, Ca^{2+} influx and p44/42 MAPK signal pathways through EGFR tyrosine kinase phosphorylation (Heo *et al*., 2006). Heparin-binding EGF (HB-EGF) could inhibit the apoptosis of mouse ESCs (Krishnamoorthy *et al*., 2009). mTeSR was superior to KnockOut-SR in supporting cell proliferation and pluripotency (Singh *et al*., 2010).

Laminins are an important and biologically active part of the basal lamina, influencing cell differentiation, migration, adhesion as well as phenotype and survival. Mouse ESCs could be grown for months on top of recombinant laminin (Miyakzaki *et al*., 2008). Recombinant laminin could be used to create a totally xeno-free and defined cell culture environment to culture human pluripotent ESCs and human induced pluripotent stem cells (Rodin *et al*., 2010). In this study, mouse ESCs were successfully isolated and cultured in glass culture dish which precoated with 10 mg L^{-1} laminin without MEFs feeder layer and the serum replaced by Knockout SR. There was no significant difference in the primary colonies formation rates and no obvious difference maximum passage number of ESCs between basal ESCs medium with MEFs feeder layer treated groups and modified ESCs medium without MEFs feeder layer and serum treated groups, respectively. But there were some differences in growth character of ESCs between those two culture systems. When the ESCs cultured in feeder layer-free and serum-free culture system, the growth of those ESCs was more slowly and doubling time was significantly prolonged, a few of ESCs were scattered in the culture dish. These experimental results researchers obtained were similar to those reported previously. Upon withdrawal of both feeder layer cells and serum, ES-S8 cells still formed colonies and expressed pluripotent markers but the colony formation efficiency, growth rate and adhesive rate were reduced which possibly resulted from the absence of factors supporting proliferation of cell and secreted from feeder layer cells such as LIF and bFGF and factors supporting adhesion of cell and from serum (Han *et al*., 2008). BG02 cells had been continuously cultured for at least 20 passages and maintained the self-renewal in feeder layer-free and serum-free culture media supplemented with bFGF, TGF-β1 and Insulin-Transferrin-Selenium (ITS) but the growth of those BG02 cells was more slowly compared with MEFs feeder, doubling time was significantly prolonged and differentiation rate of the colonies was significantly increased (Hu *et al*., 2009). Therefore, it is still necessary to improve the feeder layer-free and serum-free culture system for ESCs.

CONCLUSION

Mouse ESCs could be isolated and cultured in laminin-coated glass culture dish and DMEM supplemented with 15% Knockout SR, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 10 ng mL^{-1} LIF, 20 ng mL^{-1} bFGF, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin without MEFs feeder layer and serum and maintain their self-renewal and undifferentiated state.

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