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Immortalized mouse fetal liver stromal cells support growth and maintenance of human embryonic stem cells

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Abstract. Human embryonic stem cells (hESCs) are usually maintained in an undifferentiated state by co-culture with feeder cells. The feeder cells are important for the growth of hESCs. A novel spontaneously immortalizated mouse fetal liver stromal cell line, named KM3, was isolated from a 13.5-day mouse fetal liver. In this study, we examined whether KM3 cells could be used as feeders to support the growth of hESCs. hESCs cultured on KM3 cells showed a similar proliferation rate and characteristics to mouse embryonic fibroblasts (MEFs) after prolonged culture, including morphology, unlimited and undifferentiated proliferative ability, maintenance of normal karyotypes, formation of embryoid bodies *in vitro* and typically immature teratomas *in vivo*. Our results indicate that the immortalized KM3 cell line has the potential to support the growth and maintenance of hESCs. The cell line may be used for the large-scale expansion of hESCs in a low-cost and less labor-intensive manner.

Introduction

Human embryonic stem cells (hESCs) are derived from the inner cell masses of human blastocysts (1). The 2 basic characteristics of hESCs are pluripotency and the ability to self-renew. hESCs have the ability to differentiate into any cell type in the body. The self-renewal ability of hESCs is regulated by a set of transcription factors, including Oct-4, Nanog and Sox-2 (2). Since their derivation, hESCs hold great promise for regenerative medicine and are a powerful tool for basic research (1,3), such as disease research, toxicology and drug screening. The first hESC line was propagated in a co-culture system on a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) (1). hESCs are commonly cultured in medium supplemented with knockout serum-replacement (KSR) together with basic fibroblast growth factors (bFGF) on inactivated MEF feeders. bFGF is the key growth factor in maintaining undifferentiated growth in hESCs (4-10). bFGF has to be exogenously supplemented in the culture medium when using a mouse-feeder cell line or feeder-free conditions, as the KSR replacement medium is used instead of an animal serum in the expansion of hESCs. A number of studies have focused on the secreted factors released from MEF feeder layers, capable of maintaining the self-renewal of hESCs, and have identified a number of factors responsible for maintaining hESC pluripotency (11-14). The exact mechanisms through which feeder cells support the growth of hESCs remain only partially understood. In recent years, there have been various protocols for culturing embryonic stem cells, with the newer trends moving toward a feeder-free or serum-free culture. For human and mouse embryonic stem cells, however, fibroblast feeder layers are often used at some phase during the culture protocol. The feeders, often MEFs, provide a substrate that increases the plating efficiency, helps maintain pluripotency and facilitates both the survival and the growth of stem cells (15).

We accidentally obtained a spontaneously immortalized cell line from the mouse fetal livers in the process of utilizing mouse fetal liver stromal cells to promote the hematopoietic differentiation of hESCs. These cells appear fibroblast-like in morphology, namely the KM3 cell line. They are characterized by growing rapidly and having low nutritional requirements. We observed that the KM3 cell line does not promote the hematopoietic differentiation of hESCs. We hypothesized that the immortalized KM3 cells may be used as feeder cells for hESC culture. Thus, in the present study, we aimed to examine whether KM3 cells can support the growth of hESCs, while allowing them to retain their undifferentiated state.

Materials and methods

MEF culture and establishment of murine fetal liver-derived stromal cells. Pregnant Kunming female mice were provided

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by the Animal Experimentation Center of Jiangsu University and embryos were dissected from the uteri at 13.5 days post coitum. All experimental procedures were conducted in accordance with the Chinese legislation on animal protection. MEFs were isolated and cultured from the mouse embryos as described previously (16).

The murine fetal livers (FLs) were removed from the mouse embryos and washed with PBS 3 times, then dissected into 1-mm pieces using a scissors and treated with 0.25% trypsin/EDTA (Invitrogen) at 37˚C for 3 min. Subsequently, the cells were washed and seeded in a 25 cm^2 flask (NUNC) at a density of 5-6 FLs/flask. The culture medium contained 90% DMEM (Gibco Invitrogen) and 10% fetal bovine serum (FBS) (Gibco Invitrogen). The medium was exchanged after 24 h and 3 days thereafter. Cells were split 1:3 to 1:4 and transferred into new flasks. Usually, mouse fetal liver stromal cells are passaged only for 4-5 generations. However, the mouse fetal liver stromal cells that were derived from our research mice became spontaneously immortalized. Cell passaging lasted for 138 days from the 1st to the 5th generation and the cells showed increased proliferation from the 6th generation. The culture medium that contained 90% DMEM and 10% newborn bovine serum (NBS) (Sijiqing, Shandong, China) was changed from the 33rd passage.

hESC culture. hESCs (SHhES2) were donated by Dr Jin Ying, School of Medicine, Shanghai Jiao Tong University. The hESC culture medium contained 80% knockout DMEM, 20% KSR, 1 mM L-glutamine, 1% non-essential amino acid (NEAA), 4 ng/ml bFGF (all from Gibco Invitrogen) and 0.1 mM β-mercaptoethanol (Sigma). The cells were incubated at 37˚C in 5% CO_2 in air and 95% humidity. The hESCs were initially maintained on MEF feeders and later transferred to KM3 feeders. According to the instructions of the manufacturer, hESCs were briefly treated with 1 mg/ml of type IV collagenase (Sigma) for 30-40 min at 37˚C. The hESCs were harvested and further broken into small clumps using pipette tips and subcultured through seeding onto mitomycin C (Roche)-treated feeders (MEFs and KM3 cells) to split the cells (1:3 to 1:4). The medium was exchanged after 48 h and every day thereafter. The hESC colonies were passaged every 4 days. To assess hESC proliferation, the undifferentiated hESC colonies were counted on the MEF and KM3 feeder layer before dissociation during each of the 10 passages and then passaged in the same proportion. To determine population-doubling time, cell numbers in 5 selected independent colonies were counted under an inverted microscope. After the 20th passage, the morphological characteristics of the hESC colonies were observed with Giemsa staining.

Alkaline phosphatase and periodic acid Schiff (PAS) staining. Alkaline phosphatase (ALP) and PAS staining were carried out in 6-well plates. Prior to analysis, adherent cell layers were washed twice with PBS and air-dried. Staining was performed using Cytochemistry Staining kits (Shanghai Sun Biotech Co., Ltd), according to the manufacturer's instructions, except for staining with hematoxylin.

Karyotype analysis. For hESCs that had been cultured on KM3 cells for 47 passages, karyotype analyses were carried out. Briefly, cells were incubated with a final concentration of 0.2 μg/ml of colcemid (Sigma) of culture medium for 5-6 h. Subsequently the cells were washed twice with PBS, trypsinized in 0.25% trypsin/EDTA, treated with hypotonic 0.075 M KCl at 37˚C for 30 min, collected by centrifugation and then fixed with fresh fixative (methanol/acetic acid 3:1). After 3 rinses in fixative, the cells were dropped onto pre-cleaned chilled glass slides. After R-band staining, the chromosomes were visualized by cytogenetics specialists from the Center of Clinical Laboratory, the Affiliated Hospital of Jiangsu University.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted by TRIzol reagent (Invitrogen) from undifferentiated hESCs grown at least for 19 passages on MEFs or KM3 cells, or from control MEFs or KM3 cells. Using the ReverTra Ace kit (Toyobo), 1mg RNA was reverse-transcribed into cDNA. The PCR primers used are listed in Table I. PCR was performed by using the following parameters: denaturing at 94˚C for 5 min, 35 cycles at 94˚C for 30 sec, 60˚C (56˚C for ALP and β-actin) for 30 sec, and 72˚C for 30 sec, a final extension at 72˚C for 10 min. β-actin was used as the positive control. The PCR product was stained with 0.1 μg/ml ethidium bromide, followed by electrophoresis on a 1.5% agarose gel.

Embryoid body formation. For embryoid body formation, the undifferentiated hESC colonies were harvested by treatment with collagenase IV at the 20th passage on the MEF and KM3 feeders. The clumps of the cells were transferred onto a bacterial culture dish (NUNC). The embryoid bodies were grown in medium consisting of 80% knockout DMEM, 20% KSR, 1 mM L-glutamine, 1% NEAA and 0.1 mM β-mercaptoethanol. The medium was changed every other day. Embryoid bodies were cultured for 10 days in suspension cultures.

Teratoma formation. The potential to form derivatives of all 3 embryonic germ layers was examined in the teratomas. After 26 passages on KM3 feeder cells, approximately $2x10^6$ hESCs with undifferentiated morphology were harvested and resuspended in a mixture of PBS. The cell mixtures were subcutaneously injected into the rear legs of 4-week-old severe combined immunodeficient (SCID) mice (Laboratory Animal Center of Shanghai, Academy of Sciences, Shanghai, China). Teratomas were dissected from the mice and fixed with 4% paraformaldehyde overnight approximately 7 weeks after injection. Tumors were embedded in paraffin and histologically examined after hematoxylin and eosin staining. KM3 feeder cells ($2x10^6$) inoculated into the same sites of SCID mice as the controls did not develop tumors.

Results

Isolation and morphology of KM3 cells. MEFs and mouse fetal liver stromal cells were isolated and cultured from 13.5-day-old mouse embryos. The primary MEFs contained a heterogeneous population of cells. After 2 subsequent passages, the majority of the MEFs had a fusiform shape and were highly uniform in morphology. These cells at 2-5 passages were used as the hESC feeders. Generally, mouse fetal liver stromal cells are passaged for 4-5 generations

Table I. Information on RT-PCR primers.

Genes	Forward (For) and reverse (Rev) primers (5'-3')	Size (bp)	Annealing temperature (˚C)
Oct-4	For: TATACACAGGCCGATGTGG Rev: GTGCATAGTCGCTGCTTGA	397	60
Nanog	For: ATGCCTCACACGGAGACTG Rev: CTGCGTCACACCATTGCTA	369	60
Sox-2	For: ACACCAATCCCATCCACACT Rev: GCAAACTTCCTGCAAAGCTC	224	60
ALP	For: AGCTTCAAACCGAGATACAA Rev: ATTCTGCCTCCTTCCACC	220	56
β-actin	For: CACGAAAATACCTTCAACTCC Rev: CATACTCCTGCTTGCTGATC	265	56

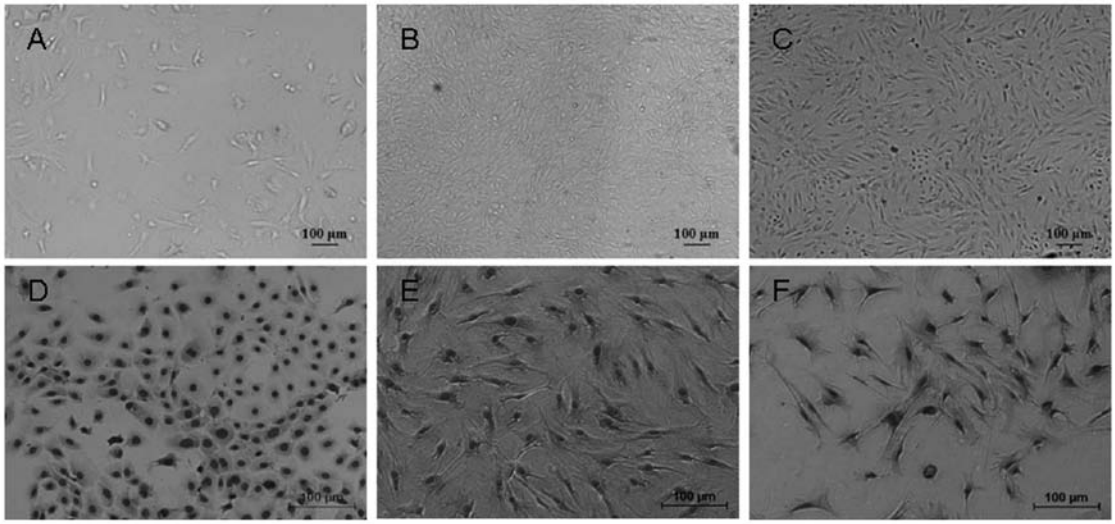

Figure 1. Morphology of mouse fetal liver stromal cell line (KM3). KM3 cells at (A) passage 1, (B) passage 24 and (C) passage 42. (D-F) KM3 and mouse embryonic fibroblasts (MEFs) were stained with Giemsa. (D) The residual epithelial-like KM3 cells after shortening the time of trypsin digestion at passage 40. (E) The purified fibroblast-like KM3 cells at passage 50. (F) MEFs at passage 3. Bar, 100 μm.

in vitro and contain a heterogeneous population of cells. We accidentally obtained a spontaneously immortalized cell line (KM3). The characteristics of KM3 cells were similar to other mouse fetal liver stromal cells before passage 4 (Fig. 1A). Nevertheless, the proliferation of the cells in passage 5 significantly decreased and reached confluence at 92 days and for cells in passage 6 at 30 days. Morphologically, the majority of the KM3 cells had a fusiform shape and were highly uniform in morphology (Fig. 1B). The interval between passages was gradually increased from 6-11 days at passages 7 to 18. The cells reached confluence every 4-5 days when they were split 1:4 at passages 19 to 24. The proliferation rate gradually increased and reached the maximum. Usually, the cells were split 1:10 and reached confluence every 4-5 days after 32 passages.

When KM3 cells were transferred to the same culture medium supplemented with NBS instead of FBS at the 33rd passage or higher, their growth rates and morphology were similar to the lines derived from FBS. KM3 cells were cultured for 132 passages. No cell senescence or reduction in the growth rate were observed. The doubling time of MEFs (passages 2 to 4) and KM3 cells (over 50 passages) was approximately 45.4 and 36.0 h, respectively. After spontaneous immortalization there were 2 cell lines of different morphologies in the culture. One cell line had a cobblestone-like morphology, similar to epithelial cells. The other resembled a fibroblast, similar to MEFs (Fig. 1C and D). In addition, we found that the fibroblast-like cells were easily digested with 0.25% trypsin/EDTA. The previously digested cells were harvested and reseeded to flasks. After approximately 50 passages, the fibroblast-like cells prevailed in population (Fig. 1E) and were similar to MEFs in morphology (Fig. 1F). This prompted us to consider whether the fibroblast-like cells may function as feeder layers for hESCs. To test this hypothesis, KM3 cells at the 50th passage or higher were treated with mitomycin C and used as feeders. We found that mitomycin C-treated KM3 cells did not proliferate but maintained their metabolic activity under the culture conditions.

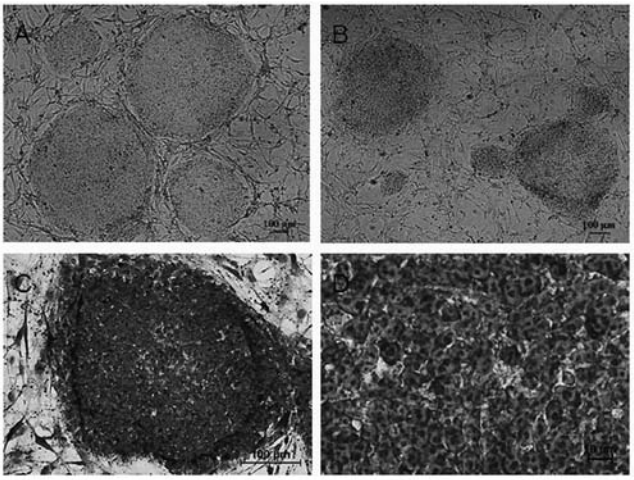

Figure 2. Morphology of human embryonic stem cell (SHhES2) colonies after 20 passages. Colonies of hESCs on grown (A) KM3 cells, (B) MEFs and (C) stained with Giemsa on KM3 cells. (D) Individual hESCs on KM3 cells retained their typical hESC morphology as shown by Giemsa staining (high nucleus/cytoplasm ratio, presence of nucleoli and spaces between cells). Bar (A-C) 100 μm and (D) 10 μm

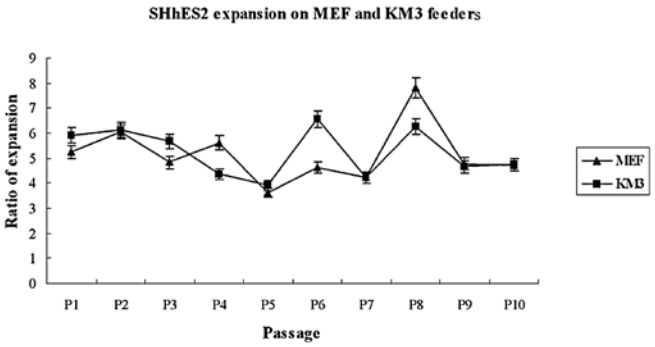

Figure 3. Ratio of expansion of hESCs (SHhES2) cultured on KM3 cells and MEFs for 10 passages. (n=5)

Morphology of hESCs. The SHhES2 cells were cultured on mitotically inactive MEFs as instructed by the provider. We cultured these hESCs continuously for 3 months and split the cells (using collagenase IV) once every 4 days. Being consistent with the provided protocol, we observed a 3-4-fold expansion in each passage. SHhES2 cells were then transferred from MEFs to KM3 cells. The density of KM3 cells was the same as MEFs ($5x10^5$/flask). We observed that hESC colonies that grew on KM3 feeder layers were slightly thinner and less compact than colonies grown on MEFs. However, the colonies had the typically undifferentiated morphology (round, defined colony edges). The slight difference in the morphology of the colonies grwon on the 2 feeders disappeared when the density of KM3 cells was increased to $7x10^5$/flask (Fig. 2A and B). The morphology of individual hESCs cultured on KM3 cells was the same as that of those cultured on MEFs. The cells appeared to be round and small, with a high nucleus/cytoplasm ratio, a notable presence of 1-3 nucleoli and typical spacing between the cells by Giemsa staining (Fig. 2C and D)

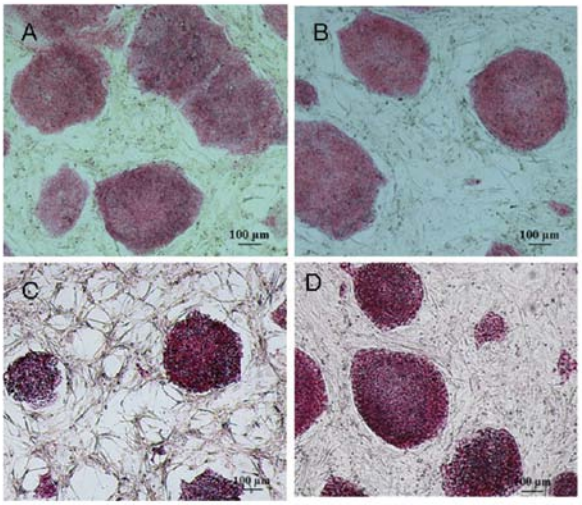

Figure 4. Cytochemical staining of hESCs (SHhES2). ALP staining of hESCs (passage 20) grwon on (A) KM3 cells and (B) MEFs. PAS staining of hESCs (passage 20) on (C) KM3 cells and (D) MEFs. The KM3 and MEF feeder cells served as the negative control. Bar, 100 μm

Proliferation rates of hESCs. To examine the proliferation rates of hESCs cultured on KM3 cells or on MEFs, the same proportion of hESCs was plated, and the number of colonies was counted before the splitting of cells in each passage. The expansion ratios of hESCs grown on KM3 cells showed no observable difference compared with those grown on MEFs, when cultured for up to 10 passages (Fig. 3). The doubling time of hESCs on MEFs and KM3 cells was 41.4 and 39.8 h, respectively. We subsequently long-term cultured hESCs on KM3. The SHhES2 line had been propagated previously and was expanded on KM3 cells for over 96 passages (approximately 380 days). The cells still showed the typically undifferentiated morphology and similar expansion ratios.

Cytochemical staining of hESCs. After the 20th passage or higher, the hESC colonies grown on MEFs and KM3 cells were analyzed by cytochemical staining. Both hESC colonies showed a strong ALP activity, while the MEF and KM3 control cells were negative for ALP expression (Fig. 4A and B). Additionally, we observed that the typically undifferentiated hESCs grown on MEFs and KM3 cells were strongly positive for PAS (Fig. 4C and D), whereas the differentiated hESCs showed a weak PAS activity, while the MEFs and KM3 cells were negative for PAS.

Stem cell marker expression in hESCs. We analyzed the gene expression levels of several stem cell markers considered to be preferentially expressed in undifferentiated hESCs by RT-PCR, such as Oct-4, Nanog and Sox-2 using the hESCs that had been cultured on either KM3 cells or MEFs for 19 passages. These results were indistinguishable from the results observed for hESCs cultured on MEFs and KM3 cells. The undifferentiated hESCs were found to be positive for ALP expression (Fig. 5)

Karyotype analysis. Karyotype analysis was performed on the hESCs cultured on KM3 cells for more than 47 passages. They

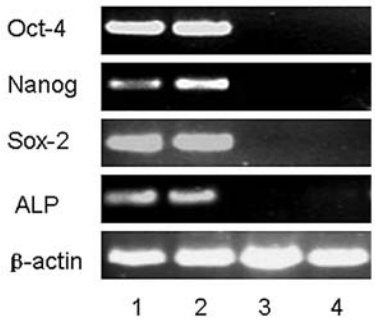

Figure 5. RT-PCR analysis results of Oct-4, Nanog, Sox-2 and ALP gene expression in the hESCs (SHhES2) cultured on MEFs (lane 1) and on KM3 cells (passage 19) (lane 2). hESCs cultured on both feeders expressed Oct-4, Nanog, Sox-2 and ALP. Lane 1, hESCs cultured on MEFs; lane 2, hESCs cultured on KM3 cells; lane 3, MEFs alone; lane 4, KM3 cells alone.

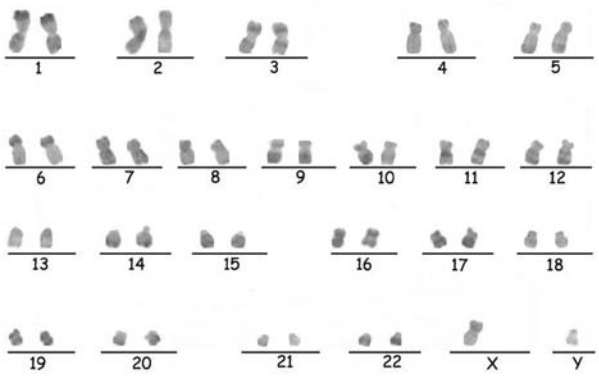

Figure 6. Karyotype analysis of hESCs (SHhES2) using the R-band method. The karyotype of hESCs cultured on KM3 cells after 47 passages was found to be 46, XY normal male.

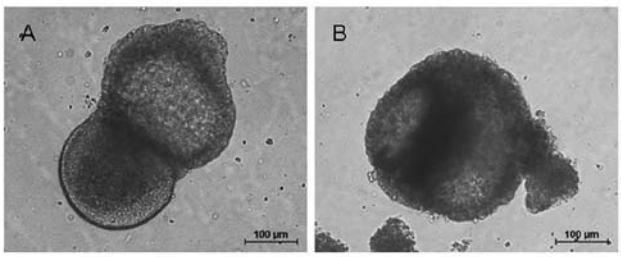

Figure 7. hESCs (SHhES2) expanded on (A) KM3 cells after 20 passages and (B) MEFs retained the potential to form embryoid bodies after 10 days in suspension cultivation. Bar, 100 μm

were found to possess the normal human 46, XY karyotype, as analyzed by R-banding. The result was compatible with the hESCs of the provider (17). The chromosomes are illustrated in Fig. 6.

Pluripotency of hESCs. To examine the pluripotency differentiation ability of hESCs cultured on KM3 cells for 20 passages, we used floating culture to form embryoid bodies. After 10 days of suspension cultivation, hESCs formed ball-shaped embryoid bodies (Fig. 7).

Additionally, the hESCs on KM3 cells for 26 passages produced typically immature teratomas *in vivo* after injection into SCID mice (Fig. 8A). The teratomas were found to contain tissues of the 3 embryonic germ layers (including immature tissues with intestinal villi, cartilages and neural rosettes), further confirming the pluripotent nature of hESCs (Fig. 8B-D).

Discussion

In this study, we describe a new immortalized cell line that can function as a feeder layer for the expansion of hESCs *in vitro*. The hESCs were cultured on KM3 cells for multiple passages, while they retained the same morphology of undifferentiated hESCs as those grown on MEFs. The hESCs were positive for ALP expression and retained a normal chromosomal karyotype. The stem cell-associated factor gene expression analysis of hESCs grown on KM3 cells showed a similarity with the cells grown on MEFs. The hESCs grown on KM3 cells successfully differentiated into embryoid bodies *in vitro* and formed teratomas *in vivo*. Furthermore, we observed that the undifferentiated hESCs were strongly positive for PAS, whereas the differentiated hESCs demonstrated a weak PAS activity. The hESCs were stably passaged for 96 passages (approximately 380 days) on KM3 cells, maintaining the morphology of typically undifferentiated hESCs. Simultaneously, KM3 cells were expanded for 132 passages (approximately 2.5 years) with medium containing 10% NBS. As feeders, they supported the hESC expansion just as efficiently as MEFs.

Several types of feeder cells have been successfully used for hESC culture. When the first hESC lines were derived, MEFs were used as feeder layers to support the propagation of hESCs in the primitive undifferentiated state (1,3). To date, hESCs have been cultured mainly by using MEFs as feeder layers. However, MEFs have many serious limitations as

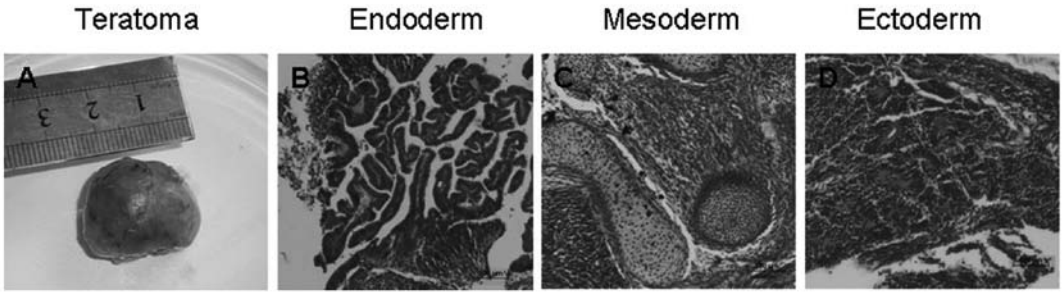

Figure 8. Teratoma formation. (A) Teratomas derived from hESCs (SHhES2) cultured on KM3 cells after 26 passages. The teratomas showed structures representative of all developmental lineages (B) intestinal villi (endoderm), (C) cartilages (mesoderm) and (D) neural rosettes (ectoderm). (B-D) Hematoxylin and eosin staining. Bar, 100 μm

feeder cells and their proliferation is limited. MEFs often go through senescence when they are passaged 5-6 times *in vitro*. Sufficient MEFs must be freshly prepared, freezed and thawed in order to fulfil the demand of the long-term and large-scale hESC culture. In addition, different batches of MEFs may differ in their growth rate and ability to support hESCs. The immortalization of feeders would offer a possible solution to this problem, resulting in a sustainable, standardized and consistent source of feeders for the culture of hESCs. On the other hand, MEFs are associated with risks, such as viral infection and transmission of mouse pathogens, which may prevent the future use of hESCs in clinical trials.

To date, scientific efforts have aimed to expand hESC-based technology from experimental research into clinical application. In order to avoid the transmission of animal pathogens, numerous human feeders from adult, neonatal and fetal sources have been used as alternative methods to culture and maintain hESCs, such as human foreskin (18-20), adult marrow stromal cells (21), human fetal muscle and adult fallopian tube fibroblasts (22,23), adult uterine endometrium (24,25), amniocytes (26-28), placenta and (29) and human fetal liver stromal cells (30). Several groups have successfully used fibroblasts differentiated from hESCs as feeders (8,31-34). These studies have shown that human cells can be used to support hESC growth and to maintain undiffierentiated hESCs. However, human fetal and adult cells may still be unsuitable feeder layers due to ethical and practical limitations. We also obtained fibroblast-like cells which differentiated spontaneously from hESCs. Nevertheless, it was difficult for these fibroblast-like cells to achieve a high passage, as they usually went through senescence and apoptosis when they were passaged 5-6 times after derivation. Since human feeder cells are unable to maintain continuity, producing sufficient hESCs for clinical therapy is difficult. Furthermore, it is important to standardize the source of feeder cells used for research, as this is a variable that could hamper comparison between results obtained by different groups.

There have been several reports describing the maintenance of hESCs on immortalized feeder layers. Park *et al* used STO, a permanently growing cell line, as a feeder to support the growth of hESCs (35,36). Several human immortalized cell lines have also been used as feeders to culture and maintain hESCs (37-39). These immortalized feeder cell lines have not been widely used by other laboratories, and more studies are required before the MEFs can be replaced completely.

The other culture systems include feeder-free (40-42), feeder-conditioned (43) and most recent suspension cultures (44). Feeder-free systems using additional growth factors will significantly increase the cost of culture. These conditions may not be optimal for a wide range of hESC lines (45). Furthermore, even though feeder-free and serum-free conditions have been defined for the maintenance of hESCs, further research is required to determine the factors responsible for maintaining the pluripotent phenotype and stability of hESC lines in general (46).

Taken together, each of these culture systems offers certain advantages and disadvantages. KM3 cells may be used as a new feeder cells to support hESC expansion *in vitro*. This has several advantages compared with other hESC culture systems. A major advantage is that the KM3 cells were immortalized and growth proceeded rapidly. The KM3 cells do not need to be freshly prepared, or frozen and thawed frequently in order to be used as a hESC feeder layer. KM3 culture only requires medium that consists of 90% DMEM and 10% NBS to highly propagate. These characteristics render KM3 cells particularly suitable for the mass production of standardized feeders to satisfy the large-scale production of hESCs, while they can decrease the cost of cultures and reduce operational stress. Moreover, our immortalized cell line was derived from mouse fetal liver tissues. Richards *et al* reported that fetal or embryonic tissues performed better *in vitro* than adult tissues when supporting the growth of undifferentiated hESCs (23). Further studies are required to determine the nature of KM3 cells and whether they can function as feeders support to other hESC lines or induced pluripotent stem cells (iPSCs). Additionally, future research is required as regards the mechanisms through which KM3 cells support hESC growth.

In conclusion, our findings suggest that KM3 cells can support the growth of hESCs (SHhES2) and may used as novel feeders for the long-term proliferation of hESCs in an undifferentiated and pluripotent state. Moreover, this expansion process has the potential for large-scale production with a simple, low-cost and less labor-intensive manner.

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