Fetal Bovine Serum Lot Selection for OP9 Cells Culture Induction System in Differentiation of Hematopoietic Stem Cells

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OP9 cell is a novel stromal cell line which had been established from new born calvaria of B6C3F2-op/op mice which lack functional M-CSF. From the original report, mice homozygous for the recessive mutation osteopetrosis (op) on chromosome 3 have a restricted capacity for bone remodelling and are severely deficient in mature macrophages and osteoclasts because the mutant mice have a mutation in the coding sequence of the M-CSF gene (Csfm gene).[1] Despite the absence of functional M-CSF activity, the level of Csfm mRNA expression remains normal. This might be due to the aberrant protein production in op/op mice.

In the past, the study of the development of hematopoietic progenitor cells in vitro from human embryonic stem cells did not produce satisfying results. Three different strategies were utilized to induce embryonic stem (ES) cells to lymphoid and myeloid precursors. The first method used was the method of embryoid body (EB) formation. In this method, ES cells were cultured with cytokines or infected with retroviruses. After a period of time, the populations of mature B, T and macrophages appeared and then they were transplanted to irradiated mice.[2] Co-cultured cells with bone marrow stromal cell lines are another method for ES differentiation.[3] The third method is a combination of EB formation and co-culturing with ES cells.[4] However, all these three methods possessed a lot of different pitfalls such as the requirement of EB formation, the need for exogenous growth factors, and differentiation into myeloids and lymphoids which appeared at different time points.

OP9 system

The OP9 culture system was developed in the year 1995 by Nakano Toru, and had several crucial strategies. First, by the removal of leukemia inhibitory factor (LIF) and other feeder cells followed by utilization of M-CSF deficient stromal cells, the M-CSF/c-fms signaling pathway was avoided. The reduction of ES cell growth by the deprivation of reducing agents such as 2-mercaptoethanol (2ME) or methyl-thyogalactoside (MTG) was avoided.[5] This system does not require any exogenous growth factors; therefore, it has been used worldwide for supporting the differentiation and induction from embryonic stem cells to blood cells and also proliferative expansion of hematopoietic cell lineages.[6-14]

OP9 which is a line of bone marrow derived mouse stromal cells has the potential ability for differentiation into adipocyte. It can differentiate into adipocyte within 2 days when given one of three adipogenic stimuli such as the insulin oleate method.[15] Not only these, but also fetal bovine serum which is heat inactivated and used in the OP9 culture system might cause OP9 cells to become adipocyte due to the different ingredients in each lot of bovine serum.[16] Therefore, testing every lot of fetal bovine serum before using the OP9 culture induction system is very essential and has the high impact on hematopoietic cell differentiation. The next section demonstrates an example experiment and methods used for selecting fetal bovine serum (FBS) lot for culturing OP9 cells.

Technique for selecting fetal bovine serum: Example experiment

Six fetal bovine serum samples from 6 companies, each with different lot numbers, were used to prepare the growth medium for OP9 cells culture. To prevent a selection bias, each lot was labelled as A,B,C,D,E, and F. OP9 cells used in this example

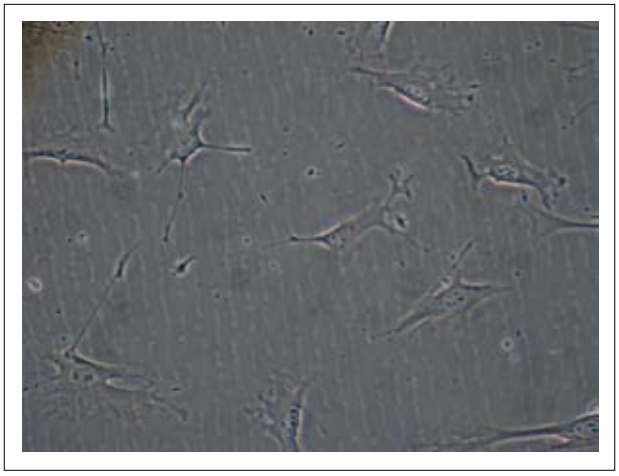

Fig 1. Morphology of healthy OP9 cells by light microscope. Adipocyte differentiation was rarely detected.

experiment were maintained in MEM-alpha (Nacalai, Japan) supplemented with 20% fetal bovine serum and 1% penicillin and streptomycin. Cells were incubated in a humidified atmosphere with 5% CO_2. For lot testing, OP9 cells were cultured in a medium containing different fetal bovine serum from each company, and subcultured for at least 2 cycles. Cells at $9x10^4$ per well were seeded into a 6-well plate using 2 wells for each lot of fetal bovine serum in order to get the 80% confluency and then they were irradiated with 50 Gy. For co-culture, human embryonic stem cells at $0.5x10^4$ cells per well were seeded on an OP9 cell feeder layer and the next day were given OP9 irradiation and were grown in human ES medium supplemented with vascular endothelial growth factor (VEGF) 20 ng/ml and this was defined as day 0. The medium was changed every other day until the collection of floating human ES cells on day 14. The morphology of healthy OP9 cells was demonstrated in Fig 1. The use of human embryonic stem cells was ethically permitted.

After culturing for 14 days, human embryonic stem cells were stained with antibodies that are indicators for well developed stem cells. In brief, the steps of the staining method were : first human ES floating cells were collected and spun at 1,000 rpm for 5 minutes at 4°C and then washed once with PBS. After that, cells were incubated with antibodies in the dark at 4°C for 30 mins. For control, the sample was next separated into 6 tubes and stained with APC (Beckman Coulter, USA) - PE (Beckman, Coulter, USA), APC-PE-Cy7 (Biole-gend, USA), PI, CD34 (BD Bioscience, USA) - CD235a (eBioscience, USA), CD34-CD45 (eBioscience, USA) and one tube as a negative control. Additionally, for other lot samples, cells were stained with CD34-CD235a, and CD34-CD45. Subsequently, all samples were further analysed by flow cytometry using a Fluorescence Activated Cell Sorter (FACS) within one hour.

FBS selection

This section shows the results from the example experiment described previously, and shows how to analyse these results for choosing the best FBS lot for further research. As shown in Fig 1, 6 samples of OP9 cells were grown in 6 different media containing different lots of fetal bovine serum. Healthy cells were seen as flat and expanded cytoplasm with rarely fatty change within the cells. The best FBS lot was selected from the analysis of hematopoietic cell induction of human ES cells by FACS. Staining with monoclonal antibodies against CD34, CD45, and CD235a revealed that lot D and E produced the highest CD45+ differentiated cells compared to other lots accounting for 21.2% and 21.1% respectively. Between lot D and E, the subgroup of CD34+ and CD45+ was found to be more abundant in lot D with the frequency at 7.8% when compared with lot E which had the lower frequency at 3.8%. Notably, lot D showed the highest expression of CD235a+ with the percentage of 31% when compared with lot E which showed only 24.4% as demonstrated in Table 1. Therefore, the fetal bovine serum lot which was considered to be the best and suitable for induction of hematopoietic stem cells was lot D.

CONCLUSION

The useful OP9 system from Japan has been established for over a decade, not only for the differentiation of specific stem cells study, but also for hematopoietic cell formation research.[17-19] Several factors were known to be secreted by healthy OP9 cells such as VEGF-C, Ang-1, interleukin-6, and interferon-3.[20] However, the selection of lot of fetal bovine serum used in OP9 stromal cells is very important. The good lot of fetal bovine serum could not only support the growth of OP9 cells, but also improve the efficacy of the OP9 induction and differentiation system.

Several specific surface markers of lymphocytic and erythroid lineages have been used in fetal bovine lot selection. For example, expression of surface markers such as CD235a, also known as glycophorin A, is a specific marker for erythroid differentiation and maturation. CD45, a pan leukocyte marker expressed in progenitor cells down regulated after erythroid differentiation, is also used to detect human embryonic stem cell differentiation in fetal bovine lot selection.

In conclusion, the best lot of fetal bovine serum is the one that could induce the best differentiation of hematopoiesis in human embryonic stem cells.

TABLE 1. Frequencies of surface marker expression on human embryonic stem cells.

Samples	Total CD45+	Total Cd34+CD45+	Total CD235a+
FBS lot A	2.5%	16.3%	17.1%
FBS lot B	3.2%	5.2%	8.5%
FBS lot C	4.2%	19.6%	9.3%
FBS lot D	21.2%	7.8%	31.0%
FBS lot E	21.1%	3.8%	24.4%
FBS lot F	2.8%	16.1%	12.8%

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