Evaluation of Pluripotency Gene Expression in Mouse Embryonic Stem Cell Cultured on the Human Feeder Layer

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ABSTRACT

Embryonic stem (ES) cells are derived from the pluripotent inner cell mass (ICN) cells of blastocysts with the potential to maintain an undifferentiated state indefinitely. The derivation process involves plating of the blastocysts on mouse embryonic fibroblast (MEF) and expansion of the outgrowth in to established ES cell line. ES cell are capable of unlimited self-renewal by symmetric division and differentiated cells to all primitive embryonic germ layers. The capacity of ES cells to differentiate in to almost all the cell types of human body highlights their potential to play a promising role in cell replacement therapies for treatment of human diseases. In this study, MEFs have been replaced with human mesenchymal stem cells (hMSCs). C4 mES cell (mouse embryonic stem cell line) colonies are cultured on inactivated hMSCs amplified ≥ 600-folds during the 30 days of continuous culture. The longest continuous expansion of C4 mES cells on hMSC was 30 passages. In this study the gene expression for Oct-4, Nanog, Rex1, Brachyury, LIF, LIFR, TERT, B2M, Stat3, Sox2, Fgf4 in mES cells using reverse transcriptase polymerase chain reaction (RT-PCR) and in which genes expression for Stat3, Sox2, Fgf4 genes was negative whilst the gene expansion for Oct-4, Nanog, Rex1, Brachyury, LIF, LIFR, TERT, B2M genes was positive. There was also a karyotype analysis for ES which showed normal result. The immunocytochemical analysis of Oct4 transcriptional factor for ES cells was made which showed positive result for this factor. These genes may be novel candidates to play critical roles in the regulation of ES Cell pluripotency and self-renewal.

Keywords: Embryonic Stem Cell; Mesenchymal Stem Cell; Blastocysts; Feeder Layer; Pluripotency

INTRODUCTION

Embryonic stem cells (ESCs) are characterized by their ability to both self-renew and differentiate [1, 2]. However, the molecular mechanisms that regulate the decision between these two processes are poorly understood. Mouse ESCs were originally isolated from the inner cell mass (ICM) of preimplantation blastocysts [3, 4] and can be maintained in cell culture indefinitely without loss of their broad pluripotent differentiation capacity as determined by their ability to give rise to all three germ layers both in vitro and in vivo [2]. The more recent establishment of human ESC lines [5] has further increased the interest in ESCs because they raise hope of an unlimited source of cells for tissue engineering and cell therapies in the future. However, realization of this potential requires an increased knowledge of the molecular mechanisms governing self-renewal and pluripotency to guide the development of processes that control the expansion and differentiation of stem cells ex vivo. Assessment of pluripotency has also relied on the expression of selected molecular markers. For murine ESCs, these have included alkaline phosphatase, the POU transcription factor Oct-4, and stage-specific embryonic antigen 1 (SSEA-1) [6]. However, the correlation between marker expression and the various functional assays has not been extensively studied. Knowledge of the intricate mechanisms regulating ESC pluripotency and differentiation potential is currently limited to a few signaling pathways (i.e., leukemia inhibitory factor [LIF]) and regulatory factors (i.e., Oct-4 and Nanog) [10, 11]. Thus, very little is known about the tolerance limits of different culture conditions for

maintaining stem cell function during expansion or how these relate to altered gene expression patterns in ESCs. Identification of molecular markers that correlate with pluripotency would be invaluable to enrich for the desired cells, as well as to monitor their maintenance during expansion protocols [10]. Achieving the goal of defining the core stem cell regulatory network requires a precise characterization of the functional capacities of the cells for which the transcriptional profile is described. In this study, we established gene expression profiles during undifferentiating of the well-defined C4m ESC line (royan C4) co-culture with hMSCs. Reverse transcription–polymerase chain reaction (RT-PCR) validation showed high correlation with the gene-array data, and several genes were also shown to have similar changes after co-culture murine ESC lines with feeder layers. In this study the gene expression for Oct-4, Nanog, Rex1, Brachyury, LIF, LIFR, TERT, B2M, Stat3, Sox2, Fgf4 in mES cells was analyzed using RT-PCR after co-culture with hMSC.

MATERIALS AND METHODS

Isolation and primary culture of hMSCs Cell solution gently overlay on to Ficoll-Hypaque, and centrifuged at 400g for 30 min at room temperature. After centrifugation, the white cell layer at the interface of the Ficoll and HBSS collected and diluted with HBSS, then it was centrifuged at 400 g for 10 min at room temperature. The isolated mononuclear cell layer was washed in PBS, re-suspended in growth medium containing Dulbecco's modified Eagle's medium (DMEM) - low glucose supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and plated in polystyrene plastic 75-Cm2 tissue-culture flasks. The cell cultures were maintained at 37°C in a humidified 5% CO2 incubator. When cells reached 70–90% confluence, cultures were harvested with 0.25% trypsin-EDTA solution.Non adherent cells were removed after 48 h. Medium was replaced every 3–4 days.

Preparation of feeder layer

Passages 2 to 40 of hMSC were used on feeder layer for C4mESC (Royan C4) cells. hMSC was first inactivated for 90 minutes regarding mitotic divisions. Then they were washed with sterile PBS three times to remove the mitomycin C (sigma-Aldrich) effect. The cells were detached from flask using 0.05% trypsin /EDTA (gibco-invitrogen) and centrifuged. Then the cell precipitate was suspended using DMED (gibco-invitrogen) and 15% FBS (gibco-invitrogen). The cells were counted using hemacytometer, and after attaching USSC to flaks bottom, their culture medium was exchanged with embryonic stem cell culture medium. This medium contains Ko-DMEM (gibco-invitrogen) , ESFCS (gibco-invitrogen) , Glutamine (gibco-invitrogen), 0.1mM β-mercaptoethanol (sigma-Aldrich), non essential amino acids, 1000u/ml ESGRO (chemicon) and 100u/ml penicillin and 100µg/ml streptomycin (gibco-invitrogen). Inactivated USSC were cultured in presence of ES culture medium to adapt to new circumstances.

Culture of mESC on human feeder layer

After preparing hMSCs as feeder layer and their adaptation to embryonic stem cell culture medium, the mESCs were brought from -80 freezers and transferred on hMSCs feeder layer. The best conditions for passage are 200-400µm diameter of mESC colonies with 45×10^8 cells. Two hours before mESC passages, the hMSCs culture medium was replaced with embryonic stem cell culture medium. Culture of embryonic stem cells requires 37℃ and 5%CO2. Their culture medium should be changed on a daily basis.

Karyotype analysis for mESC

Before and after co culture, mESCs were subject to karyotype analysis. The first and last passages were preferably chosen for karyotype analysis. The cells were first incubated with 0.1µg/ml colcimid for 3 to 4 hours. Then the cells were trypsinized and 0.075 M Kcl solutions were added to them and incubated in 37℃ with 5% CO2 for 20 minutes. Then 3:1 methanol and acetic acid were added for fixing the samples. Then the cells were spread on the lamella surface and the chromosomes were subject to karyotypic analysis.

Immunocytochemical analysis

mESC cultured on hMSC feeder layer was used for immunocytochemical analysis. The cells were first placed in 4% Para formaldehyde. They were put in 4℃ for 30 minutes and then in room temperature for another 30 minutes. After twice washing with cold PBS, 4 percent triton was added to them. After 40 minutes in room temperature, the cells were washed using 1% PBS/Tween. The cells were then placed in blocking solution containing 1 percent HSA/PBS

for 30 minutes. After two times of washing, primary antibody of Rabbit anti mouse Oct-4 was added and kept in 4℃ for 12 hours away from light. After three times washing with 1 percent PBS/tween, secondary antibody (PE-conjugated) was added to cells with a 1:30 ratio. After three hours they were washed with PBS/tween and Dapi was used to stain the nuclei.

Total RNA isolation and RT-PCR

RT-PCR was used to evaluate the expression of Oct-4, Nanog, Rex1, Brachyury, LIF, LIFR, TERT, B2M, Stat3, Sox2; Fgf4. Total RNA was isolated from cells using RNA extraction kit. Prior to reverse transcription (RT) RNA samples were digested with DNase I to remove contaminating genomic DNA. Standard RT was performed using Reverse RevertAid TM First Strand cDNA Synthesis Kit and 2 µg total RNA, 0.5 µg oligo (dt18) per reaction, according to the manufacture's instructions. PCRs were conducted using approximately 50 ng cDNA to amplify a number of marker genes. After initial denaturation at 95ºC for 1 min, PCR amplification was continued at 95ºC for 40 s, annealing temperature for 40 s, and 72ºC for 1 min for a total 30 cycles, and final extension at 72ºC for 5 min. Amplified PCR products were separated on a 2% agarose gel electrophoresis and stained with ethidium bromide.

RESULTS

Morphology of mESC

mESC maintained their continues proliferation potential on human feeder layer. C4 mESC underwent more than 30 passages successively and remained in undifferentiated form. mESCs have typical morphology on MEF feeder layer including high N/C ratio, clear nucleolus dense cells and lack of space between cells. Morphology of mESC on USSC is completely similar to mESC on MEF and has the same typical morphology (figure 1).

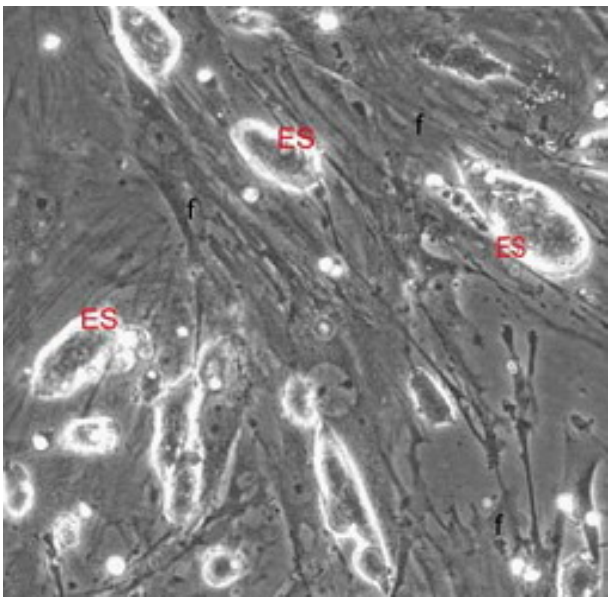
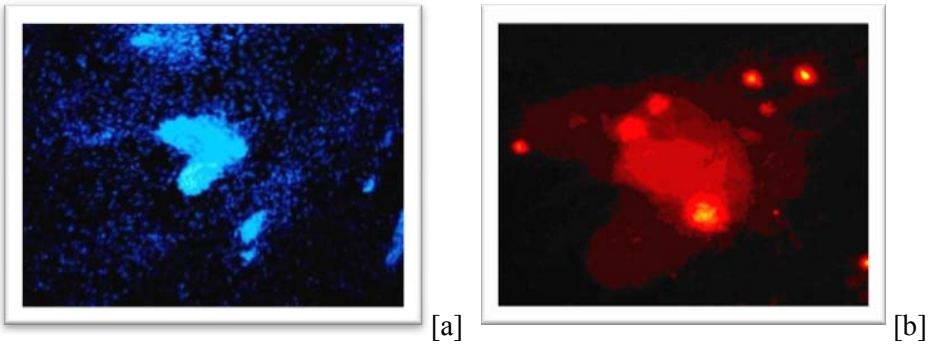


Figure 1: morphology of mES cell colonies culture on hMSC. mESCs were continuously cultured

Immunocytochemical analysis

Monoclonal antibodies against Oct-4 were used to investigate the expression of the inner cell mass expanded cells in culture medium. The expression of this marker was detected in C4 ES cells as positive control. The percentages of Oct-4 positive cells were 50± 6% in cells. This marker did not express in MSCs as control group (data not shown) whereas Nucleus staining with DAPI show strong signal in both c4 mES and hMSC cells (figure 2).



[a] [b]

Figure 2.. Immunostaining of mES cell colony with anti-oct4 [a]. Nuclei were stained with DAPI in the same cell[b].

Expression of pluripotent cell specific Markers

In this study β-globulin was used as negative control. Mouse embryonic stem cell cultures on human feeder layer were evaluated for pluripotency genes. RT–PCR for Oct-4, nanog, TERT, LIF, Brachyury, Rex-1, BMP4 and B2M was positive. RT–PCR was not done for ES cells undergoing ten passages. Therefore, it was shown that all ES cells on hMSC feeder layer are in undifferentiated state and the hMSC have effectively acted as feeder layers. To determine if the gene expression changes observed in the C4 ESCs are general and more broadly observed, the expression Patterns of 11 genes were followed after co-culture with hMSCs using quantitative RT-PCR. Specifically, RT-PCR analysis was able to verify the expression changes of genes such as *SOX2*, *Stat3*, and *FGF4* that showed significantly decreased transcript in C4 mESC line tested. The RT-PCR results also confirmed that *Oct-4, Nanog* transcript levels were not decreased significantly during any passages in C4 mESCs. Decrease in *LIF* transcripts was significant in C4 mESCs according to the gene array; the RT-PCR results indicated a reduction (figure 3).

Karyotype analysis for m ESC

Before beginning the experiments, mESCs were also subject to karyotype analysis before experiment and it was shown that they possess normal chromosomal karyotype of 40xx. After more than 20 passages these cells were again analyzed for karyotype and it was indicated that they possess normal chromosomal karyotype of 40xx. Figure 3 shows chromosome karyotype of m ESC (figure4).

DISCUSSION

The present study is distinct from previous studies in two important aspects. The first and the most important, only a selected population of germ line–competent ESCs, grown under carefully controlled, optimized culture conditions, was used to establish the gene expression profiles. It is likely that substantial variation in gene expression arises in response to culture variables. Considering the multiplicity of culture variables that can be important for the biological heterogeneity of cell populations and their gene expression profiles, remarkably little information has been provided about the conditions used to generate the cells for the gene expression profiles reported thus far [7–8, 9]. Second, few of the previous studies addressing questions about a shared or common stem cell gene expression signature among different types of stem cells, or even different ESC lines, have involved correlative functional assays to assess the pluripotency and self-renewal capacity of the cells of interest [12, 13]. By combining the gene expression profiling data with assays measuring ESC pluripotency and self renewal, it should be possible to more precisely define the genes critical for specifying these properties [14, 15]. We based the present evaluation on gene expansion changes which can be influenced by culturing and ES preservation conditions. The gene expression for Oct4, Nanog, Rex1, TERT, LIF, LIFR, Stat3, Sox2, Fgf4, Brachyury genes in C4 mES cells which were in the co-culture conditions with hMSCs was evaluated. Feeder layer has an important role in preservation and up keeping of ES cells in undifferentiated condition. The feeder layer generally maintained ES cells in undifferentiated conditions by releasing multiple cytokines, such as LIF. Feeder layer cells produce a large range of cytokines including LIF some of which are still unknown and with no clear mechanism. In this study we aimed on surveying gene expression of C4 mES cells in co-culture conditions with hMSC and generating gene profiles from C4 mES that specifies whether C4 mES's being co- culture to hMSc has any influence on existing gene expression pluripotency of ES.

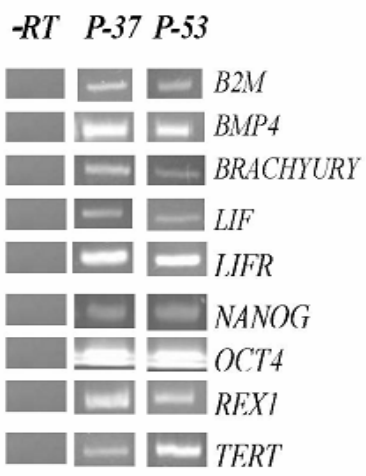


Figure 3. Expression of Stat3, BMP4, REX1, Nanog, Brachyury, TERT, LIF, LIFR, and Fgf4 were evaluated by RT-PCR.

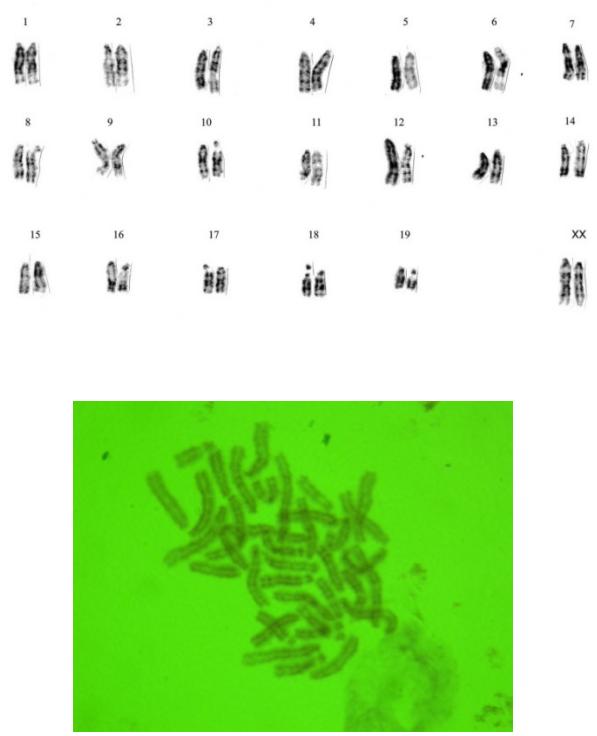


Figure 4. Karyotype analysis of mESCs expanded on human feeder cell. C4-mESC culture on hMSC feeder cells represented normal 40XX karyotype

Important roles in the maintenance of undifferentiated ESCs have previously been demonstrated for several of the down regulated factors such as *Stat3*, *Rex1*, *Sox2*, *Gbx2*, and *Bmp4* [10, 11]. The results show that Stat3, Sox2 and *Fgf*4 genes in C4 mESC that were co-culture to hMSC are not expressed; on the other hand, BMP4 has positive expression. In previous studies there were reports of pathway including: Oct4, Sox2, which in cooperation help maintain undifferentiated Es cells whereas in this study only Oct4 expression was positive and Stat3, Sox2 had no expression. There are two probabilities: first, Oct4 maintains self-renewal condition in the new pathway solely or by cooperating with other factors; second, conditions might be exclusive to C4 mES cells. Our result suggests that: the transcription factors and Nanog are capable of inducing the expression of each other, moreover, they are essential for maintaining the self-renewal undifferentiated of C4 mEScs. These two genes can participate in different pathways solely or cooperatively and maintain the pluripotency in ES cells. Induced pluripotent stem cells, from a non-pluripotent cell, typically and adult somatic cell, by inducing a "forced" expression commonly abbreviated as ips cells or iPSCs, are type of pluripotent stem cells artificially derived of certain genes. The importance of these results is where to specify all of Oct4, Nanog genes, and their functions and make them powerful candidates for being placed in iPS. As forced Oct4 expression induces pluripotency inSox2-null cells, a group of researchers concluded that the primary role of Sox2 in induced pluripotent stem cells is controlling Oct4 expression, where as in this study Sox2 has not been expressed.

A detailed understanding of these molecular mechanisms will thus be essential for developing human ES cells as in vitro model systems for studying embryonic development and for harnessing the differentiation potential that makes them highly attractive for cell-based therapies. In this study, we used a novel strategy to identify genes that may play a critical role in regulating the pluripotent potential of mouse ES cells.

The result of the present study shows that C4 mESC can keep its undifferentiated condition well in co-culture with hmsc, and the pluripotency of gene expression for *Oct4*, *Nanog, TERT, BMP4, LIF, LIFR, branchyury and Rex1* were positive and for *Stat3*, *Fgf4*, *Sox2* genes it was negative. *Brachyury* and *LIF* genes were stricken with down regulation during passages and, *BMP4*, *TERT, LIFR; Rex1* gene was stricken with up regulation. *Oct4*, *Nanog*, genes had a steady expression during different passages. These genes may be novel candidates to play critical roles in the regulation of ESC pluripotency and self-renewal. Taken together, this work provides the foundation for achieving a greater understanding of the molecular mechanisms that govern and reflect the capacity of ESCs for multilineage differentiation and self-renewal.

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