A specific and quick gene expression study in mouse ES cells

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Abstract

Purpose To study gene expression at single embryonic stem cell colony levels with a new RT-PCR protocol.

Methods Forty-five mouse ES cell colonies were retrieved at the 5th, 10th, 15th, 20th and 25th passages. The pluripotent state was analyzed for OCT-4 and Nanog, and β -actin as a control for the presence of templates. RT-PCR was done using the SuperScript™ III CellsDirect cDNA Synthesis System. Every 2 or 3 days just before passage, a single colony was loaded into a 0.5 ml PCR tube containing 10 μ l of resuspension buffer using a pulled glass pipette.

Results The RT-PCR protocol was completed in less than 150 min. All colonies were positive for OCT-4 and β -actin and 42 out of 45 were positive for Nanog.

Conclusions This protocol requires as little as 10 pg of total RNA starting material and is therefore useful for low cell number tissues, such as single stem cell colonies or preimplantation embryonic materials.

Keywords ES cells · Gene expression · RT-PCR

A gene expression study at the single ES cell colony level is described which may improve previous procedures that use materials from pooled colonies.

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Introduction

Although stem cell research has currently been one of the hottest fields in biomedical sciences, the definition of a stem cell remains largely, due to some technical difficulties, functional. Knowledge of the intricate mechanisms regulating ES cell pluripotentiality and differentiation potential is currently limited to a few signaling pathways and regulatory factors [1]. As ES cell colonies are typically established together with feeder layers, their growth may be affected by intercellular/intracellular communication, resembling a similar period during embryogenesis. In fact, murine ES cells closely resemble cells of the late inner cell mass (ICM) of the blastocyst in their cell-surface antigen phenotype [2].

Different technologies are available for expression studies of gene transcripts [3]; however these protocols require multistep RNA extraction that can lead to the loss of material. Conventional ES cell gene expression protocols also require large amounts of material for gene expression studies; a few micrograms of total RNA are needed for cDNA synthesis during reverse transcription and PCR amplification [4–7]. It has been estimated that a mouse oocyte has about 0.4 ng total RNA and a day 3.5 embryo (~64-cell blastocyst) has about 2 ng total RNA [8], which may explain in part the necessity of working with pools of colonies. Furthermore, data from average over cell populations can overlook critical information within a single colony.

To overcome this limitation, an RT-PCR protocol was evaluated for mouse ES cell gene expression analysis in single colonies. The purpose of this study was to evaluate a new possible RT-PCR protocol for ES cell diagnosis at the single colony level over many passages, requiring smaller amounts of total RNA starting material and to simplify the

Fig. 1 Mouse ES cell isolation with PMEF. Days of culture: day 1 (**a**), day 3 (**b**), and day 5 (**c**). ES cell colony at third passage (**d**). ICM (1) and trophoblast (2)

procedure by using a single tube for all reactions. We chose two genes, Oct-4 and Nanog that have already been tested in previous studies [5, 9].

Analyzing a single mouse ES cell colony would then diminish the risk of detecting RNA transcripts that actually come from contaminating cells of different colonies when studying them together in a pool of colonies.

Materials and methods

Blastocysts from 129S1/SvImj mice were cultured one per well in 24 well dishes previously prepared with gelatin coating using ultra pure water with 0.1% gelatin (Specialty Media, Phillipsburg, NJ, www.specialtymedia.com). Each well contained 1.0 ml of ES cell medium which consisted of 750 ml of Dulbecco's modified Eagle's medium-DMEM, low bicarbonate formulation; Fetal Bovine Serum (20%); 2 mM L-Glutamine; 50 µg/ml Penicillin/Streptomycin; 0.1 mM MEM Non-Essential Amino Acids; 10 ml Nucleosides; 0.1 mM Beta-Mercaptoethanol (all from Specialty Media), and 2,000 U/ml of leukemia inhibitory factor (LIF) (Chemicon International; Temecula, CA; www.chemicon.com). ES cell medium was stored at 4°C and utilized for up to 7 days.

Prior to use, medium was warmed and equilibrated in an incubator for at least 4 h, at 37°C, 5% CO₂. The media was changed daily and after 5 to 6 days of culturing the blastocyst, inner cell mass outgrowths (ICM-O) were washed once with Ca⁺⁺/Mg⁺⁺-free PBS (Specialty Media).

ES cell lines were isolated from 129S1/SvImj mouse blastocysts using methodology similar to that described by Nagy et al. [10]. Fifty-five ICM-Os were used during de novo ES cell isolation with two different procedures. The first procedure used 37 ICM-Os (group I) previously cultured in gelatinized dishes prepared with 1×10^5 mitotically arrested primary mouse embryonic fibroblasts (PMEF) per well (Specialty Media). The second procedure used 18 ICM-Os (group II) previously cultured in gelatinized plates without PMEF. The ICM-Os were removed using a Pipetman P-200 and then incubated, one ICM-O per well for 4 min at 37°C with 0.25% trypsin/1 mM EDTA (Specialty Media) in a 96 well dish. After neutralizing trypsin with an equal volume of ES medium, ICM-Os were disaggregated using the Pipetman and then returned to their previous wells in the initial 24 well dish with new ES cell medium.

Every 2 to 3 days the cells of plated ICM-O were submitted to trypsin treatment by incubating for 4 min at

Fig. 2 Mouse ES cell isolation without PMEF. Days of culture: day 1 (**a**); day 3 (**b**); and day 4 (**c**). ES cell colony at third passage (**d**). ICM (1) and trophoblast (2)

37°C with 0.25% trypsin/1 mM EDTA after washing with one change of $\text{Ca}^{++}/\text{Mg}^{++}$ -free PBS. Each passage was recorded in a log book to keep control of cell culture.

After de novo isolation and expanding into 12 well dishes, sequential biopsies were taken at 5th, 10th, 15th, 20th and 25th passages for RT-PCR (Figs. 1 and 2) from

each nine newly isolated ES colony, including a total of 45 ES colonies with good morphology.

Using a pulled glass pipette biopsy was performed, each one consisting of a whole single ES cell colony removed under a dissecting microscope just before trypsin treatment and loaded into a 0.5 ml PCR tube containing 10 μl of

Table 1 Description of primers used in RT-PCR

GSP*	Primers 5'–3'	AT (°C)	Product size (bp)	Position on cDNA	Tm (°C) (50 mM NaCl)	Related sequences	References
β -Actin	For-CAA GGT GTG ATG GTG GGA ATG G	55	408	43–64	59.5	<u>M12481</u>	Krussel et al. [20]
	Rev-CAG GAT GGC GTG AGG GAG AGC A			450–429	64.5		
OCT-4	For-GGC GTT CTC TTT GGA AAG GTG TTC	55	313	537–560	60.0	<u>BC068268</u>	Hay et al. [9]
	Rev-CTC GAA CCA CAT CCT TCT CT			849–830	54.8		
Nanog	For-AGG GTC TGC TAC TGA GAT GCT CTG	55	364	184–207	59.9	<u>AY278951</u>	Chambers et al. [5]
	Rev-CAA CCA CTG GTT TTT CTG CCA CCG			547–524	61.0		

*GSP Gene specific primers, OCT-4 and Nanog multipotent state markers.

Table 2 ES cell de novo isolation rate with and without PMEF

Group	Blastocysts	New colonies	Percentage (%)	<i>p</i> Value
I—With PMEF	37	8	21.6	0.12
II—Without PMEF	18	1	5.5	

resuspension buffer, a solution provided by the RT-PCR kit. Each colony was loaded into a PCR tube carrying approximately 1 μ L of ES cell medium into the RT-PCR reaction.

RT-PCR protocol

SuperScript™ III CellsDirect cDNA Synthesis System (Invitrogen, Carlsbad, CA, www.invitrogen.com) was used for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Lysis and reverse transcription were performed in the same tube and products were treated with DNase I to eliminate genomic DNA. All the procedures were done while the tubes were on ice. After each step the reaction was mixed by gently pipetting and briefly spinning to collect the contents. One microliter of Recombinant Ribonuclease Inhibitor (40 U/ μ L-RNaseOUT™) was added to each tube and the tubes were immediately stored at -20° C until the RT-PCR procedure.

After collecting the biopsies over a period of 2 months, PCR tubes were thawed, 1 μ L lysis enhancer was immediately added, and the tubes were then transferred to the thermal cycler (Biometra, Applied Biosystems, Foster City, CA) at 75° C for 10 min, then submitted to DNase I treatment with 5 μ L DNase I, Amplification Grade (1 U/ μ L) and 1.6 μ L 10 $\times$ DNase I Buffer, and incubated for 5 min at room temperature.

Following the DNase treatment, 1.2 μ L of 25 mM EDTA was added to each tube on ice and submitted to a new period of incubation at 70° C for 5 minutes. Each tube from the DNase I digestion received 2 μ L of Oligo (dT) 20

(50 mM) and 1 μ L of 10 mM dNTP Mix and then was incubated again at 70° C for 5 min. Tubes were placed on ice for 2 min.

Six microliters 5 $\times$ RT Buffer, 1 μ L RNaseOUT™, 1 μ L SuperScript™ III RT (200 U/ μ L) and 1 μ L 0.1 mM DTT were added to each tube before incubated for 50 min at 50° C. The reaction was inactivated at 85° C for 5 min. One μ L of RNase H (2 U/ μ L) was added to each tube which was then incubated at 37° C for 20 min. The reactions were chilled on ice and stored at -20° C until PCR amplification.

PCR

A master mix was prepared for a final 50 μ L PCR reaction with the following components: 5 μ L 10 $\times$ PCR Buffer, 1 μ L 10 mM dNTP, 1.5 μ L 50 mM $MgCl_2$, 1 μ L 10 mM of each primer, 2 μ L cDNA from RT-PCR reaction, 0.4 μ L of Platinum® *Taq* DNA Polymerase (2.0 U) and 38.1 μ L of autoclaved Milli-Q water.

Tubes were centrifuged and incubated in a thermal cycler at 94° C for 1 min to denature the template and activate the enzyme. Forty cycles of PCR amplification were performed as follows: denaturation at 94° C for 30 s, annealing at 55° C for 39 s and extension at 72° C for 53 s. PCR primers that have been proven to work in previous publications (Table 1) were used.

RT-PCR and PCR were run with negative controls and 10 μ L of PCR products were analyzed by 2% agarose gel electrophoresis. Zona pellucida free embryos were

Fig. 3 PCR results from single colony mouse ES cells. Controls (C): blastocyst without zona pellucida for Oct-4 and Nanog; mouse liver total RNA (50 ng/ μ L) for β -actin). Negative RT control without Reverse Transcriptase (RTc). Hela total RNA positive control (*Hela*)

used as a positive control to access the gene expression of Oct-4 and Nanog; β -actin was used as a control for the presence of templates.

Results

A comparison was made between groups with and without PMEF (Table 2) after the 3rd passage when new ES cell lines could be clearly recognized and isolated. Of 37 blastocysts in group I, eight new ES cell lines were isolated (21.6%) and one ES cell line out of 18 blastocysts (5.5%) was isolated from group II ($p=0.12$) (Figs. 1 and 2).

As we removed one colony at 5th, 10th, 15th, 20th and 25th passages from each new ES cell line, we could study a total of 45 colonies: 40 colonies from group I and five colonies from group II. The entire RT-PCR procedure was performed in the same tube and the protocol was completed in less than 150 min. All 45 single mouse ES cell colonies showed positive gene expression for Oct-4 and β -actin (Fig. 3). Forty-two out of 45 ES cell colonies were positive for Nanog.

Discussion

Gene expression studies of stem cells using microarray have generated tremendous amounts of information [11–13]. Nevertheless, one limitation of this approach is that it is currently impossible to purify stem cells to absolute homogeneity. Genes expressed by contaminating differentiated cells may therefore be detected in the stem cells samples [14]. However, for obtaining specific information for each gene, RT-PCR is still one of the most useful methods. Our study clearly indicated that RT-PCR can provide specific profiling of a particular gene of interest at the single colony level.

One would ask what the best limit is for gene expression study: single cell [15], single colony or pool of colonies [11–13]. Although this answer depends on outcome measures and study design, it seems reasonable to believe that ES cell colonies do not grow up together as a group of hundreds of cells by chance. There might be intercellular/intracellular communications that drives their culture into units or colonies; and morphological similar colonies may have different expression profiles. We decided to study specific gene expression at the single colony level and to examine if overlooking gene expression information might occur using a pool of colonies.

Most commercial kits require a few micrograms of total RNA as starting material for cDNA synthesis during reverse transcription and for PCR amplification, and these procedures tend to be lengthy. Unlike most articles that describe RT-PCR protocols using total RNA extracted from

pools of colonies, which may not represent the actual status of each of every single colony, the protocol describes here seems to be effective for studying gene expression within a single colony. Furthermore, the protocol may even be applicable to study gene expression in single cells, which contain as little as 10 pg total RNA, a sensitivity level suggested by the manufacture.

In addition, the whole RT-PCR procedure is fast and has been simplified by the use of a single tube for all reactions, without intermediate steps for organic extractions or ethanol precipitations. This advantage is indicated by the fact that three out of 45 colonies did not show expression of Nanog, which could not be revealed if materials from a pool of colonies were used. It was recently reported that specific populations within feeder-free human ES cell cultures can be isolated and expanded using fluorescence-activated cell sorting. Different surface expression of SSEA-3 with cells from the same ES cell line was demonstrated [16].

More research with single ES cell colonies would be useful as a way to assess single clone gene expression with more purified and homogeneous cells. This may be a better approach to be used in future microarray studies, as currently genes expressed by contaminating cells may also be detected while using total RNA from pools of colonies [14].

Ivanova et al. described the molecular similarities and differences among five distinct stem cell populations whose similarities have been called “stemness” [11], nevertheless, there are many questions about the best definition of ES cells. Some markers have been used to access the multipotent state by RT-PCR, as Oct-4 and Nanog. Nanog is a homeodomain protein that directs propagation of undifferentiated ES cells and its mRNA is present in pluripotent mouse and human cell lines, and absent from differentiated cells. In preimplantation embryos, Nanog is restricted to cells from which ES cells can be derived. Expression of Oct4 overlaps with that of Nanog both before and after the window for stem cell derivation [5].

With this sensitive protocol, we found 3 of 45 colonies derived from Group II that expressed Oct-4, failed to express Nanog. Further study is needed to determine whether or not these three colonies that are negative for Nanog are different in their “stemness”, or if they are losing their undifferentiated status. An alternative explanation may be that Nanog is dispensable, as a recent paper suggested that the induction of pluripotent stem cells from mouse embryonic or adult fibroblasts under ES cell culture conditions was implicated by four factors, Oct-3/4, Sox2, c-Myc, and Klf4, but not Nanog [17].

Since the first report on successful isolation of mouse stem cells [18], numerous studies on methodology improvement have been published. Today, procedures for establishing mouse stem cell lines are routinely performed

in laboratories around the world and detailed steps are described in many text books. Nevertheless, the culture conditions required for isolation and maintenance of ES cell status have yet to be fully understood.

In the present study, we also noticed a few technical details that may improve isolation and establishment of stem cell lines. Most protocols call for mouse ES cells to be isolated and cultivated with PMEF. Our preliminary observations indicated ES cells with morphological and molecular markers of an undifferentiated state were maintained up to the 25th passage (about 60 days) in gelatinized plates with 2,000 IU/ml of LIF and Fetal Bovine Serum (20%) in ES cell medium without PMEF. Whether or not subsequent passages would produce similar results is unknown since the culture was stopped at the 25th passage and the cell colonies were frozen to conduct RT-PCR studies. While isolating ES cells, we compared the *de novo* isolation rate of two different groups, i.e., the use of PMEF during the first isolation procedure in group I and without using PMEF in group II. It is known that PMEF produces LIF [10], which is important for maintaining the multipotent state. Our results showed a higher percentage rate for ES cell isolation in the PMEF's group (21.6% vs 5.5%), however the difference between the groups was not statistically significant. Further studies are required to test the outcome of increased LIF concentrations in the culture medium.

Most previous studies utilize a glass pipette with a diameter no greater than the cell clump to disaggregate the ICM-O clump into smaller cellular aggregates of three or four cells [10]. We, however, obtained 9 new ES cell lines (21.6%) by pipetting up and down with a Gilson P200 pipetman after trypsinization of ICM-O; this procedure was substantially easier. The difficult approach described in some protocols for ES cells *de novo* isolation may explain in part why approximately two thirds of all mouse ES cell original research published between 1981 and 2001 feature combinations of only six key cell lines and subclones [19]. Although we didn't compare the use of glass pipettes with Gilson P200 for disaggregating colonies, we would recommend Gilson P200 as it appears easier than the former method for disaggregating colonies into cell solutions.

Conclusion

The protocol described here features sensitivity and simplicity for gene expression profiling of stem cells. The number of cells required is small as starting material and the entire RT-PCR procedure is rapid and has been simplified by the use of a single tube for all reactions without intermediate steps for organic extractions or

ethanol precipitations. This approach can be useful for gene expression studies within a single colony as an alternative to most RT-PCR protocols that require total RNA extracted from pools of colonies, which may not represent the actual status of every single colony.

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