ESGRO-2i Medium: Inhibitor-Based Serum-free Medium for ES and iPS Cell Culture

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Abstract

Defined serum-free and feeder-free culture of mouse embryonic stem (mES) cells holds many advantages over the classical serum-containing feeder-dependent culture methods, ranging from decreased lot-to-lot variations to ease of culture. In this study, we explore the use of inhibitor-based, serum-free and feeder-free ESGRO-2i medium (EMD Millipore) for culturing mES cells and induced pluripotent stem cells (iPS cells).

Introduction

The discovery that the inhibition of differentiation inducing signals is critical for mES cell self renewal even in absence of the cytokine Leukemia Inhibitory Factor (LIF) led to a new definition of the ground state of ES cell self-renewal[1]. Inhibitors that block MAPK/Erk pathway, in combination with the glycogen synthase kinase 3 (GSK3) inhibitor, protect mES cells from differentiation-inducing signals, allowing for self-renewal in serum-free medium[2,3]. However, mES cells maintain their LIF responsiveness[1,4].

Materials and Methods

Immunocytochemistry: For Oct-4 staining, we used mouse Oct-4 antibody (Santa Cruz) at a 1:100 dilution, and secondary donkey anti-mouse IgG-FITC (EMD Millipore, Cat. No. AP129F) antibody at a 1:500 dilution.. For the SSEA-1 staining, we used mouse SSEA-1 antibody (EMD Millipore, Cat. No. MAB4301) at a 1:50 dilution, and secondary goat anti-mouse IgM-FITC (Jackson Laboratories) antibody at a 1:500 dilution.

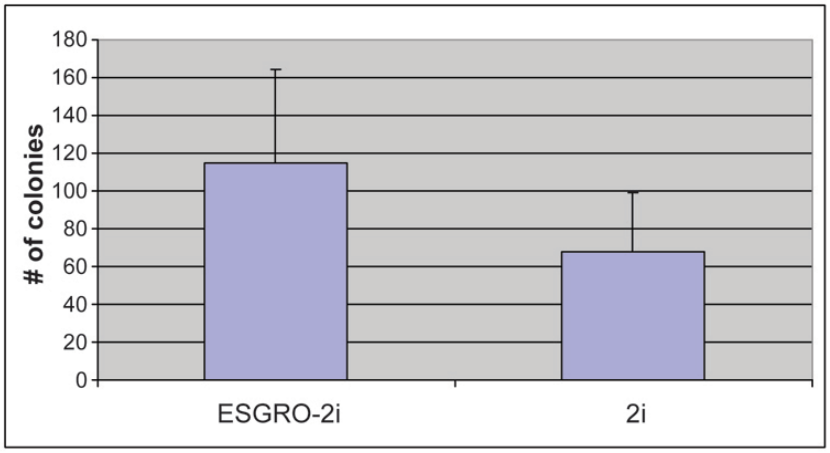
Low density clonal assay

mES cells were expanded for 1-2 passages prior to low density clonal assay in ESGRO Complete Plus Clonal Medium (EMD Millipore, Cat. No. SF001). Cells were split with Accutase™ reagent (EMD Millipore, Cat. No. SCR005), washed twice with ESGRO basal medium (EMD Millipore, Cat. No. SF002) and counted. 6-well plates were coated with 0.1% gelatin, and wells were seeded with 1000 cells/well in either ESGRO-2i medium or in 2i medium (without LIF). After 5 days, colonies were counted.

Two-step RT-PCR with Amplifluor® primers:. mES cells and induced pluripotent stem cells (iPSCs) were cultured in a 6-well plate until confluence. Total RNA was extracted using the RNeasy kit (QIAGEN). Samples were treated with RNase-free DNase prior to reverse transcription. cDNA was synthesized from total RNA with oligo(dT) and random hexamer primers mix provided with the iScript™ cDNA Synthesis Kit (Bio-Rad). 1 µg of total RNA was used in 20 µL of reaction. The reverse transcription reaction consisted of 25°C for 5 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes. 2 µL of the reverse transcription reaction was used in PCR amplification. PCR reactions were carried out using STEMCCA™ Viral Gene Detection qPCR Multiplex Kits (EMD Millipore, Cat No. SCR581). Briefly, the multiplex qPCR reactions were carried out using TITANIUM™ Taq DNA polymerase (Clontech) with a final concentration of 6 mM $MgCl_2$ and 300 µM dNTP. The PCR reaction consisted of an initial denaturation at 95°C for 3 minutes followed by 45 cycles of 30 seconds at 95 °C and 30 seconds at 60 °C. Positive plasmid controls from the kits were used to generate template standard curves along with the unknown cDNA samples for later quantitative determination of the relative copy number of the samples. Expression levels of the viral Oct-4 and endogeneous Nanog were normalized to the housekeeping GAPDH gene.

Results

During the development of ESGRO-2i medium, we first compared 129SvEv mES cell propagation with a low density clonal plating assay in the presence of LIF (ESGRO-2i) with propagation in the absence of LIF (2i). We found that the presence of LIF in the inhibitor medium led to better colony propagation and greatly enhanced the general health of ES colonies, confirming that pluripotent cells remained LIF-responsive (Figure 1).



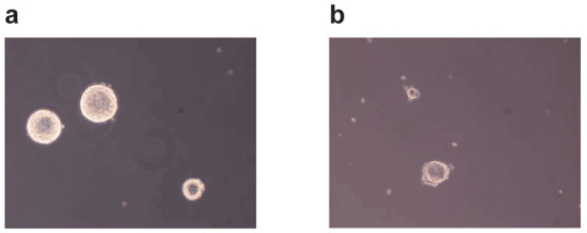


***Figure 1. LIF responsiveness of 129SvEv mESCs**. Emerging colonies from the low density clonal assay were counted and morphology documented. a) colonies in ESGRO-2i which contains LIF, GSK3b inhibitor, and Mek1/2 inhibitor. b) colonies in 2i which contains basal medium with GSK3b inhibitor, and Mek1/2 inhibitor.*

After culturing 129SvEv mES cells for more than 10 passages in ESGRO-2i medium, cells were injected into C57Bl6 host blastocysts which yielded two high-percentage and one medium-percentage chimeras (Table 1).

foster mother	# of blastocysts	# of cells injected/blastocyst	live births	chimeras	% chimerism
1	11	8-15 cells	3	1	90
2	10	8-15 cells	no pregnancy	n/a	n/a
3	10	8-15 cells	1	0	n/a
4	10	8-15 cells	3	2	90, 60

***Table 1. Chimera generation**. 8-15 129SvEv agouti mESCs were injected into C57Bl6 blastocysts and a total of 41 blastocysts were transferred to four donor females. Chimerism was judged according to percentage of agouti coat color. Chimeras are currently being bred for germ line transmission.*

We analyzed the pool of injected mES cells for pluripotency markers, both by staining (Figure 2) and by quantitative RT-PCR and found that relevant markers were expressed. Strikingly, one of the pluripotency markers, mouse nanog transcript (mNanog), underwent a 6-fold up-regulation in mES cells in ESGRO-2i medium when compared to mES cells cultured in parallel in serum-containing medium on a feeder layer (data not shown), suggesting that nanog upregulation is the mechanism by which ESGRO-2i medium supports pluripotency.

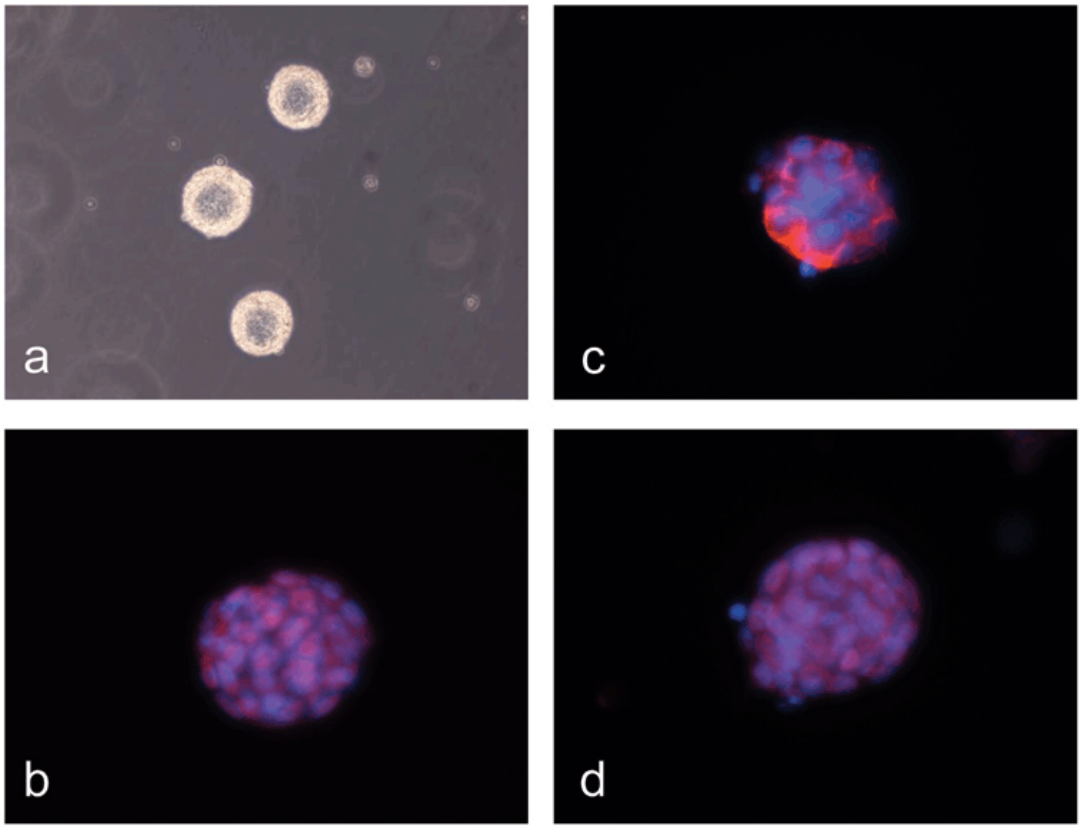


Figure 2. Prolonged pluripotent culture in ESGRO-2i*. 129SvEv mESCs were cultured for 10 or more passages. Pluripotency was assessed a) morphologically using bright field microscopy, b) with anti-Oct4 antibody staining, c) with anti-SSEA antibody staining, and d) with anti-Sox2 antibody staining.*

We then tested ESGRO-2i medium for maintenance of pluripotency of induced pluripotent stem (iPS) cells. Currently, reprogramming is thought of as process by which cells revert through a primed state (pre-iPS cell) before they reach the naïve pluripotent state. These distinct states of pluripotency can be specified by culture conditions and are characterized by morphology, signaling pathway dependency, and epigenetic signatures. We hypothesized that ESGRO-2i medium might provide the culture conditions to aid reversion of pre-iPS cells to the naïve state. To test this hypothesis, we generated several iPS cells with the STEMCCA polycistronic (OKSM) reprogramming lentiviral vector (EMD Millipore).

We then expanded iPS cells both in ESGRO-2i medium and in serum-containing medium on feeder layers. To measure the induction of pluripotency, we measured endogenous nanog expression as well as viral transgene expression represented by lentiviral Oct4 transcripts after 3-5 passages (early passage) and after 10 or more passages (late passage). We found that endogenous nanog up-regulation was concomitant with viral transgene down-regulation, after extended iPS cell culture in ESGRO-2i medium. In contrast, neither up-regulation of nanog nor down-regulation of viral Oct4 expression was observed in iPS cells cultured in serum-containing conditions on feeder layers (Figure 3). This suggested that ESGRO-2i medium aids in acquisition of naïve pluripotency and may be used to rescue partially reprogrammed iPS cells to a fully reprogrammed, naïve pluripotent state.

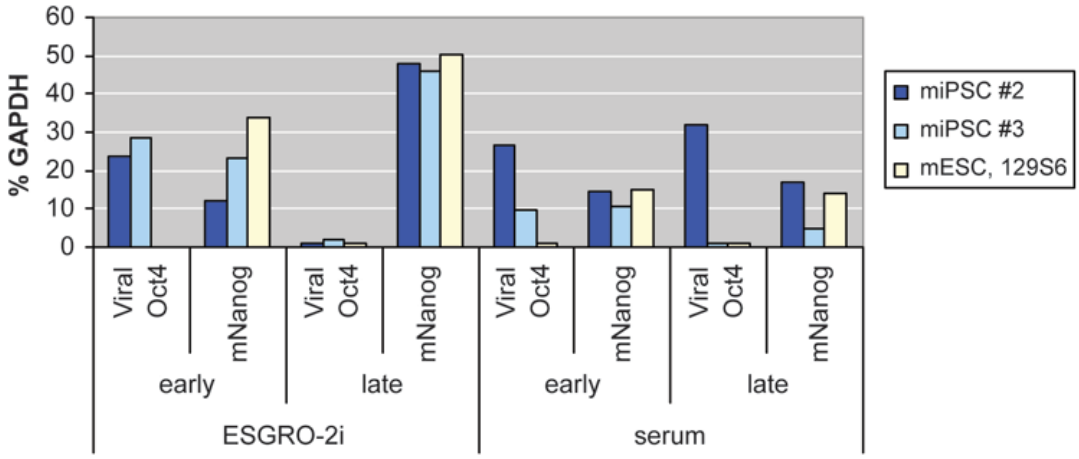


***Figure 3*. Nanog and Viral Oct4 expression in iPSCs.** *Viral Oct4 and mNanog transcript levels are shown in 129S6 mESCs at early passages (3-5), and at late passages (>10) in parallel cultures containing ESGRO-2i medium or serum containing medium in the presence of feeder layers.*

Moreover, we found two iPSC clones with morphological features of epiblast stem cells (EpiSCs), such as low clonogenicity, and low adherence in both culture conditions (data not shown). These clones had relatively high viral Oct4 and lower nanog transcript levels, suggesting a partially reprogrammed state. After prolonged culture in ESGRO-2i medium but not after culture in serum-containing medium, we observed emerging adherent colonies with mES-cell-like morphological features, suggesting that ESGRO-2i medium could provide a favorable culture condition for selection of naïve iPS colonies. Further studies are being performed to validate this observation.

Conclusion

Recently, LIF/2i medium was shown to provide a favorable environment for transitioning pre-iPS cells to naïve iPS cells[5]. This transition was accompanied by increased endogenous nanog expression while viral transgene expression was down-regulated, highlighting the role of nanog in facilitating acquisition of pluripotency[6]. This is consistent with our results, supporting evidence that maintenance of pluripotency in the defined ESGRO-2i medium is mediated at least in part by nanog.

In summary, we show that the defined serum-free and feeder-free ESGRO-2i medium can be used for prolonged pluripotent mES cell maintentance and naïve iPSC culture.

References

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