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Esg1, expressed exclusively in preimplantation embryos, germline, and embryonic stem cells, is a putative RNA-binding protein with broad RNA targets

Tetsuya S. Tanaka,[1] Isabel Lopez de Silanes,[2] Lioudmila V. Sharova,[1] Hidenori Akutsu,[1] Toshiyuki Yoshikawa,[1] Hisayuki Amano,[3] Shinya Yamanaka,[3] Myriam Gorospe[2] and Minoru S. H. Ko[1,*]

[1]*Laboratory of Genetics and* [2]*Laboratory of Cellular and Molecular Biology, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224, USA; and* [3]*Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan*

In our earlier attempt to identify genes involved in the maintenance of cellular pluripotency, we found that KH-domain protein *Embryonal stem cell-specific gene 1* (*Esg1*) showed similar expression patterns to those of *Oct3/4* (*Pou5f1*), whereas the forced repression of *Oct3/4* in mouse embryonic stem cells immediately downregulated the expression of *Esg1*. Here we further confirm this overlap by *in situ* hybridization and immunohistochemical analyses. Both *Esg1* transcript and protein exist in the egg and preimplantation embryos. At embryonic day 3.5, blastocyst stage, however, ESG1 protein was more abundant in the inner cell mass (ICM) than in trophectoderm (TE), whereas *Esg1* transcript was detected in both the ICM and the TE, particularly in the polar trophectoderm. The presence of an RNA-binding KH-domain in ESG1 led us to search for and identify 902 target transcripts by microarray analysis of immunoprecipitated ESG1 complex. Interaction of 20 target mRNA with ESG1, including *Cdc25a*, *Cdc42*, *Ezh2*, *Nfyc* and *Nr5a2*, was further validated by reverse transcriptase–polymerase chain reaction of the immunoprecipitation material, supporting the notion that ESG1 is an RNA-binding protein which associates with specific target transcripts.

Key words: cellular pluripotency, *Esg1*, immunoprecipitation-microarray, RNA-binding protein.

Introduction

Mouse embryonic stem (ES) cells (Evans & Kaufman 1981; Martin 1981) have been widely used to study the biological functions of genes by targeted mutagenesis. Furthermore, the ES cell is a good model system to understand mechanisms of cellular pluripotency and differentiation, because ES cells can be maintained in an undifferentiated state indefinitely *in vitro* by leukemia inhibitory factor (LIF), but can be induced to differentiate into a variety of cell types. Constitutive activation of the transcription factor *Stat3* (Matsuda *et al.* 1999), a downstream effector of LIF signaling, and the homeobox gene *Nanog* (Chambers *et al.* 2003; Mitsui *et al.* 2003) have been demonstrated to be sufficient to maintain the undifferentiated state of ES cells independently without LIF. Another transcription factor *Oct3/4* (*Pou5f1*; Niwa 2001; Cavaleri & Scholer 2003) has been demonstrated to play a central role in the formation and maintenance of pluripotent stem cells, including the inner cell mass (ICM) of blastocysts (Nichols *et al.* 1998; Boiani *et al.* 2002) and ES cells (Niwa *et al.* 2000), and germ line (Kehler *et al.* 2004; for a review see Boiani & Scholer 2005). In addition, other transcription factors such as *Sox2* (Avilion *et al.* 2003) and *Foxd3* (Hanna *et al.* 2002) are involved in these processes, but only *Oct3/4* (Pesce & Scholer 2001; Cavaleri & Scholer 2003) and *Nanog* (Chambers *et al.* 2003; Mitsui *et al.* 2003) showed very restricted expression patterns in preimplantation embryos, undifferentiated ES cells, and germline cells.

In our earlier attempt to identify genes involved in the maintenance of cellular pluripotency, we compared the global expression profiles between mouse ES cells and trophoblast stem (TS) cells by microarrays (Tanaka *et al.* 2002). We showed that *Embryonal*

*To whom all correspondence should be addressed.
Email: komi@mail.nih.gov
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stem cell-specific gene 1 (*Esg1*) is a candidate for such a gene. *Esg1* was first identified as a gene whose expression was downregulated when embryonic carcinoma (EC) cells were induced to differentiate (Astigiano *et al.* 1991), and as a gene expressed differentially between undifferentiated ES cells and a differentiated parietal yolk sac cell line (Bierbaum *et al.* 1994). The comparison of global gene expression profiles singled out *Esg1* as having the greatest measured expression difference between ES and TS cells (Tanaka *et al.* 2002). Other microarray analyses of stem cells (Ivanova *et al.* 2002; Ramalho-Santos *et al.* 2002) and analyses of GenBank expressed sequence tag (EST) frequency (named *Dppa5*, Bortvin *et al.* 2003; *Ecat2*, Mitsui *et al.* 2003) also revealed *Esg1* as specific to ES cells. The forced repression of *Oct3/4* in mouse ES cells immediately downregulates the expression of *Esg1* (Tanaka *et al.* 2002). Western *et al.* (2005) have recently reported the spatiotemporal expression pattern of *Esg1* transcript and protein in embryos and germline.

Here we report further expression profiles of *Esg1* mRNA and protein, the microarray-based identification of ESG1 target RNA molecules, and the expression patterns of these target RNA in *Esg1*$^{-/-}$ ES cells.

Materials and methods

A more detailed description is available from Tanaka *et al.* (2006).

Collection of mouse embryos

C57BL/6J, and B6D2F1/J (C57BL/6J x DBA/2 hybrid), and CD1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and from the Charles River Laboratory (Wilmington, MA, USA), respectively. Collection of eggs, preimplantation embryos, and *in vitro* fertilization were performed as described (Szczygiel *et al.* 2002; Tanaka & Ko 2004).

Northern hybridization

FirstChoice Mouse Blot I (Ambion, Austin, TX, USA) and Mouse Embryo Stage Blot (Seegene, Seoul, South Korea) were hybridized with a radiolabeled cDNA of *Esg1* in an Ultrahyb (Ambion Inc., Austin, TX) as previously described (Tanaka *et al.* 2002).

In situ *hybridization*

Digoxigenin-labeled sense or antisense RNA probes for *Esg1* and *Oct3/4* were processed essentially as described (Tanaka *et al.* 2002). For preimplantation embryos, hybridization was done on the Transwells in 24-well plates (Corning Coster, Cambridge, MA, USA) as previously described (Yoshikawa *et al.* 2006).

Immunological detections

Affinity purified anti-ESG1 polyclonal antibody raised against ESG1 with GST fused in frame (αESG1 Pab) was used for immunoblot and immunocytochemistry as described already (Tanaka & Ikenishi 2002). For whole-mount staining, αESG1 Pab or rabbit preimmune serum was used in combination with either TROMA-1 (Hybridoma Bank, University of Iowa, Iowa City, IA, USA), anti-α-tubulin monoclonal antibody (Sigma, St Louis, MO, USA), or anti-β-actin monoclonal antibody (Sigma). As secondary antibodies, tetramethylrhodamine isothiocyanate (TRITC)-conjugated antirabbit IgG (Sigma) and either fluorescein isothiocyanate (FITC)-conjugated antirat IgG (Sigma) or FITC-conjugated antimouse IgG (Sigma) were used. Stained embryos were observed by the DeltaVision system (Applied Precision, Olympus, Tokyo, Japan).

Immunoprecipitation array analysis

Putative protein–RNA complexes were pulled down by either αESG1 Pab- or rabbit IgG-conjugated protein-A beads (Sigma) from precleaned ES cell lysate (Lopez de Silanes *et al.* 2004). Radiolabeled cDNA synthesized from pulled-down RNA were hybridized onto M17K arrays (National Institute on Aging 2003; Tanaka *et al.* 2000; VanBuren *et al.* 2002). Data extracted by the Array Pro software (Media Cybernetics, Silver Spring, MD, USA) were normalized by Z score transformation. Twenty of the identified ESG1 targets were further validated by quantitative polymerase chain reaction (Q-PCR). The complete dataset and a list of primer pairs used for validation are available as supplemental materials (Tanaka *et al.* 2006).

Quantitative polymerase chain reaction analysis

Total RNA extraction from intact ES cells, *Esg1*$^{+/-}$ and *Esg1*$^{-/-}$ ES cells (Amano *et al.* 2006), cDNA synthesis and Q-PCR were performed as previously described (Tanaka *et al.* 2002). The list of primer pairs used is available as supplemental materials (Tanaka *et al.* 2006).

Results

Expression profile of Esg1 *transcript*

To investigate the localization of *Esg1* transcripts, we performed northern blot analysis and found *Esg1*

transcripts (~0.7 kb) in adult ovary and to a lesser extent, in testis, but not in other adult organs (Fig. 1A) or in embryos at stages from embryonic day (E) 4.5–18.5 (data not shown). *In situ* hybridization detected *Esg1* transcripts in the cytoplasm of growing germinal vesicle (GV)-stage oocytes in the sections of adult ovary (Fig. 1B), but not in those of newborn ovary (which contains no GV-stage oocytes), or testis (data not shown). Because of the absence of the detectable signal in testis by *in situ* hybridization, a larger, relatively abundant band (~2 kb) detected in testis by northern blot may be a result from

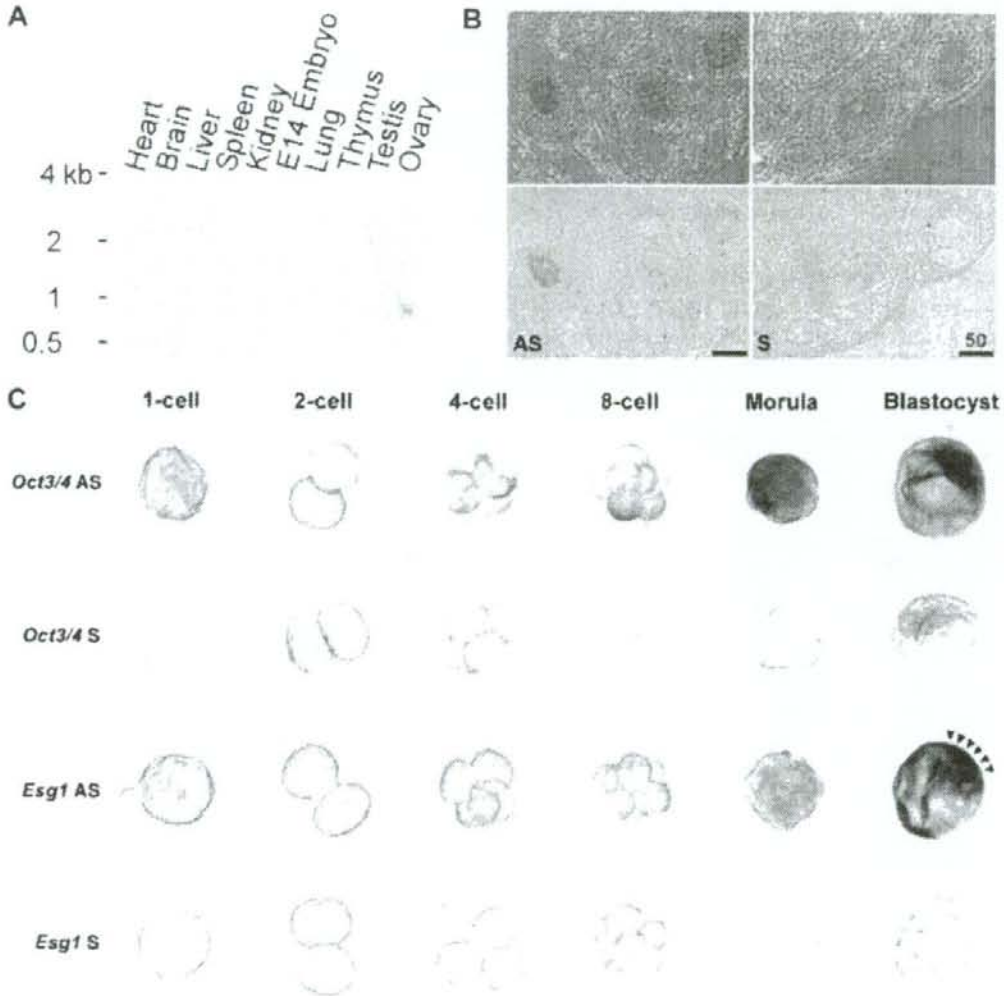

Fig. 1. Expression patterns and localization of *Esg1* transcript. (A) Northern hybridization analysis showed a ~0.7 kb transcript of *Esg1* in adult ovary and to a lesser extent, in testis. (B) *In situ* hybridization on the section of adult ovary. Phase contrast images are shown on the top. AS, antisense probe for *Esg1*; S, sense probe. Bars, 50 μm. (C) Whole-mount *in situ* hybridization (WISH) on preimplantation embryos. WISH was repeated three times and gave almost identical results. Only representative embryos are shown. Stages of embryos examined are indicated on the top of the panel. In blastocysts, the expression of *Esg1* was detected both in the inner cell mass and the trophectoderm (black arrowheads), whereas no expression of *Oct3/4* was detected in the trophectoderm (white arrowheads).

cross-hybridization of a probe (Fig. 1A). Whole-mount *in situ* hybridization (WISH) analysis of preimplantation mouse embryos detected *Esg1* transcripts in the cytoplasm of unfertilized eggs (data not shown) and individual blastomeres of 1-cell, 4-cell, 8-cell, morula, and blastocyst stages (Fig. 1C). Sense probes for either *Esg1* or *Oct3/4* gave no detectable signals (Fig. 1B,C). This expression pattern was similar to that of *Oct3/4*, with the exception that although *Esg1* transcripts were present in both the ICM and trophectoderm (TE), *Oct3/4* transcripts were absent from the TE (Fig. 1C). These results are consistent with reverse transcriptase–polymerase chain reaction (RT–PCR) analyses on microdissected ICM and TE (Tanaka *et al.* 2002). More mature blastocysts at E4.5, however, showed predominant expression of *Esg1* in the ICM (data not shown; see Western *et al.* 2005).

Expression profile of ESG1 protein

A polyclonal antibody was raised against ESG1 for localization studies (αESG1 Pab), whose specificity was confirmed by immunoblotting with purified recombinant ESG1 (data not shown), or crude lysate of ES cells (Fig. 2A; see also Fig. 3C). Immunofluorescent microscopy using the αESG1 Pab detected ESG1, like its transcripts, in the cytoplasm of GV-stage oocytes in the section of adult ovary (Fig. 2B, right). This was confirmed independently by whole-mount immunofluorescent microscopy of isolated oocytes with the DeltaVision microscope (Fig. 2B, left). The localization of ESG1 did not change significantly during the completion of the second meiosis (Fig. 2C), although the signal was relatively reduced in the area surrounding metaphase II chromosomes (animal hemisphere) compared to the opposite side of eggs (vegetal hemisphere; see arrowheads in Figure 2D). In preimplantation embryos, the staining of ESG1 was comparable in the cytoplasm of all blastomeres up to morula stage (Fig. 2d), but was weaker in the outer cell-layer of late-morula and in the TE of blastocysts (cf. the co-staining of TROMA-1 antibody recognizing the EndoA cytokeratin specifically localized in the TE and primitive endoderm (Brulet *et al.* 1980) (Fig. 2D)). The discrepancy in the ESG1 localization in the TE between Western *et al.* (2005) and our study may be ascribed to the differences of fixation methods utilized (4% paraformaldehyde vs methanol + dimethylsulfoxide) and the developmental stages observed (E4.5 vs E3.5). We noted a speckled staining pattern in the cytoplasm of unfertilized eggs and in the individual blastomeres of preimplantation embryos (Fig. 2D). Preimmune serum gave no signal at any stage of preimplantation embryos. Thus, *Esg1* transcript was detected in the cytoplasm of almost all blastomeres and both the ICM and the TE in early blastocysts, whereas the ESG1 protein was rather restricted to the central region of eggs and embryos, and to the ICM of blastocysts.

The expression of *Esg1* in primordial germ cells (PGC), suggested earlier by the presence of EST in PGC-specific cDNA libraries (Tanaka *et al.* 2002), is now shown unequivocally in PGC in gonads and *in vitro* derived embryonic germ (EG) cells (Western *et al.* 2005). It is thus established that the expression of *Esg1* is restricted to cells in the pluripotent cycle, where *Oct3/4* and *Nanog* had previously been the only genes that showed this idiosyncratic expression pattern.

Identification of RNAs that bind to ESG1

Considering the fact that ESG1 has a KH-domain, we hypothesized that ESG1 was an RNA-binding protein and associated with a subset of target RNA molecules. To test this notion, we first immunoprecipitated ESG1 from undifferentiated ES cells (ESG1-IP) using the anti-ESG1 antibody described above and employed methodologies that preserved ribonucleoprotein complexes. RNAs present in the immunoprecipitation material were then isolated from the complex and used for microarray hybridizations. RNAs isolated from the complexes immunoprecipitated using a non-specific IgG from rabbit serum were also hybridized as a control (Fig. 3A; Lopez de Silanes *et al.* 2004). Hybridization was done in triplicate with three separate preparations of RNA for both ESG1-IP and control IgG-IP. Out of 16 896 genes on the microarray (8418 unique; Tanaka *et al.* 2000; VanBuren *et al.* 2002), 902 transcripts were found to be enriched significantly in the ESG1-IP following the criteria described previously (Lopez de Silanes *et al.* 2004). Among these, 207 genes corresponded to well-characterized genes, including genes related to cell cycle (*Ccnd3*, *Ccnf*, *Cdc25a*, and *Cdc42*), chromatin remodeling (*Ezh2*, *Fancg*, *Mta3*, *Sca10*, *Sin3a*, and *Smarca4*), transcription factors (*Gata3* and *Sox13*), and *Esg1*. To validate the results obtained by immunoprecipitation (IP)-microarray analysis, we designed primer pairs for 24 genes of interest and performed RT–PCR on RNA prepared in the same manner as for IP-microarray analysis. Although three primer pairs failed to amplify any products, the abundance of the remaining transcripts, including *Esg1* mRNA itself, was enriched in the ESG1-IP compared with the IgG-IP (Fig. 3B). The αESG1 Pab detected only a single band (~12 kDa) in the immunoblot of the IP materials (Fig. 3C), further establishing the specificity of the IP results. Taken

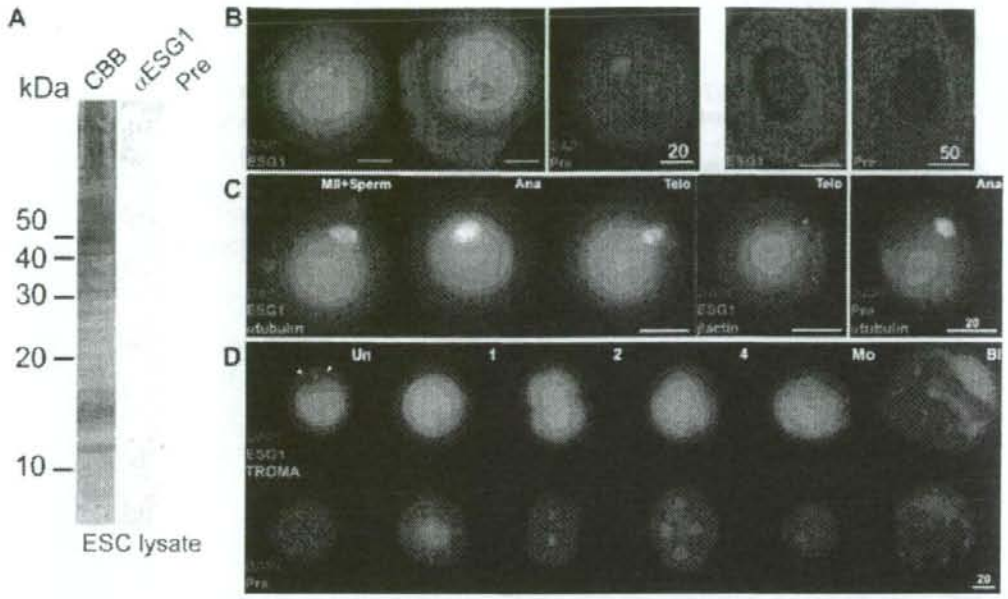

Fig. 2. Expression patterns and localization of ESG1 protein. (A) Crude lysate from embryonic stem (ES) cells was run on a 12.5% sodium dodecyl sulfate–polyacrylamide gel, followed by either CBB staining (CBB), or immunoblotting with αESG1 Pab (αESG1) or preimmune serum (Pre). A single band with a molecular weight of ~12 kDa was recognized by αESG1 Pab. (B–D) Immunofluorescent microscopy to localize ESG1 in oocytes and preimplantation embryos. In each panel, DNA was stained with 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI, blue), and tetramethylrhodamine isothiocyanate (TRITC)-conjugated antirabbit IgG antibody (red) was used to detect the presence of αESG1 Pab or preimmune serum. The experiment was repeated at least twice and only representative results are shown. (B) Localization of ESG1 in the cytoplasm of germinal vesicle (GV)-stage oocyte. (B, Left) Microdissected GV-stage oocytes stained with either αESG1 Pab (ESG1) or preimmune serum (Pre) on whole-mount. Only single optical sections of embryos are shown. Note that cumulus cells surrounding a GV-stage oocyte on a middle panel have more condensed DNA than in GV-stage oocyte so that the sensitivity of the DAPI channel was higher in the left and the right (Pre) oocytes than in the middle oocyte. Bars, 20 μm. (B, Right) Sections of adult ovary stained with either αESG1 Pab (ESG1) or preimmune serum (Pre). Bars, 50 μm. (C) Localization of ESG1 at stages of fertilization (MII + Sperm), the second anaphase (Ana), and the second telophase (Telo) as indicated above. Eggs were co-stained with αESG1 Pab (ESG1, red) and either anti-α-tubulin monoclonal antibody (α-tubulin; green) or anti-β-actin monoclonal antibody (β-actin; green). All of the optical sections with 1 μm thickness each (40–50 sections/egg) were projected together on single planes to visualize a whole chromosomal structure. An egg stained with preimmune serum and anti-α-tubulin monoclonal antibody at the second anaphase stage is shown as a negative control. Bars, 20 μm. (D) Localization of ESG1 in preimplantation embryos at stages of unfertilized eggs (Un), 1-, 2-, 4-cell embryos, morulae (Mo) and blastocysts (Bl) as indicated above. Embryos stained with preimmune serum are shown on the bottom. TROMA-1 monoclonal antibody was used in order to distinguish trophectoderm. White arrowheads indicate the area where less-intense staining of ESG1 was detected. Bar, 20 μm.

together, these results support that ESG1 is associated with RNA that encode proteins with a broad range of functions.

To further study the RNA-binding properties of ESG1, we performed pull-down analyses using biotin-labeled target RNA transcribed *in vitro*. Following incubation with recombinant ESG1, the complex was affinity-purified using streptavidin-coated beads and ESG1 was detected by western blotting. However, the binding ability of recombinant ESG1 to its targets appeared to be weak in the absence of other possible (as yet unknown) interacting factors present in ES cells (data not shown). Therefore, it remains to be investigated whether ESG1 binds to RNA directly.

Altered RNA levels of ESG1 target in Esg1$^{-/-}$ *embryonic stem cells*

In order to ascertain whether ESG1 influenced the steady-state levels of its target transcripts, we quantified the abundance of ESG1 target RNA in *Esg1*$^{+/-}$ and *Esg1*$^{-/-}$ ES cells by Q-PCR (Fig. 3D). This analysis

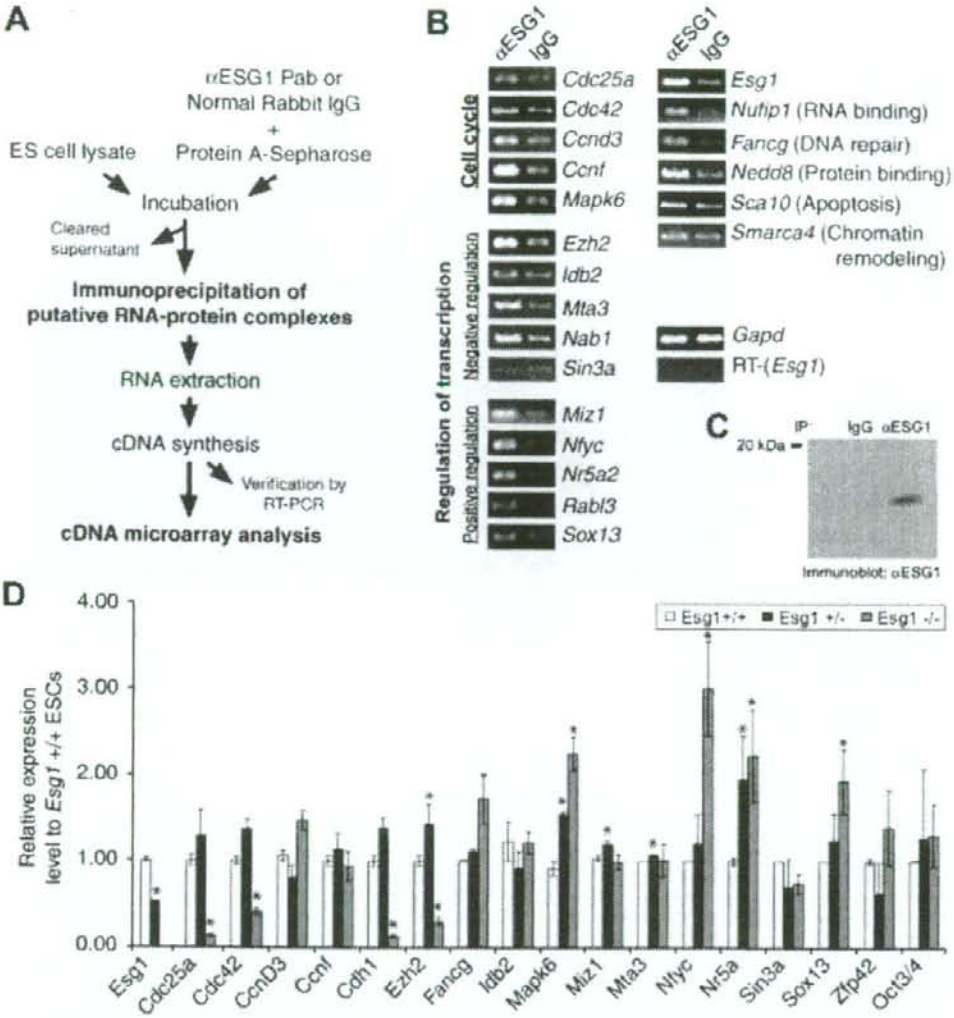

Fig. 3. Immunoprecipitation-microarray analysis identified binding targets of ESG1. (A) Schematic representation of immunoprecipitation (IP) followed by microarray analysis. See text for details. (B) Validation of IP-array results shown in (A). Relative to the control (IgG), enrichment of RNA that were immunoprecipitated as protein-RNA complexes by αESG1 Pab (αESG1) was confirmed by reverse transcriptase–polymerase chain reaction (RT–PCR) for 20 genes with the IP materials prepared independently. Of 20 genes, 15 were classified into three groups according to their putative functions (left). Putative functions of the rest of the genes were also indicated (right). Interestingly, ESG1 bound to *Esg1* RNA itself. GAPDH (*Gapd*) was used as a loading control. No PCR product for *Esg1* was detected from RT-negative reaction (RT–). (C) The IP materials (IgG and αESG1) separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were processed for immunoblotting with the anti-ESG1 antibody. (D) Quantitative PCR analysis revealed that expression levels of some of the binding targets of ESG1 were altered in *Esg1*$^{-/-}$ ES cells. Relative expression levels to the intact ES cells (*Esg1*$^{+/+}$) for each gene are shown. Although same sets of genes shown in (B) were examined, the results of only representative genes are shown. The rest of the genes did not show any detectable alteration of their expression levels (data not shown). Expression levels of marker genes such as *Cdh1*, *Zfp42*, *Oct3/4*, were also examined. For intact *Esg1*$^{+/+}$ ES cells, results of three biological replications were averaged. For *Esg1*$^{+/-}$, results of three biological replications from two independent lines (clones #1, #33) were averaged and for *Esg1*$^{-/-}$, results of three biological replications from three independent lines (clones #2, #7, #27) were averaged. Asterisks (*) indicate statistically significant differences (ANOVA, $P < 0.05$) of gene expressions between intact *Esg1*$^{+/+}$ and *Esg1*$^{+/-}$ ES cells and between intact *Esg1*$^{+/+}$ and *Esg1*$^{-/-}$ ES cells.

revealed that many ESG1 target genes showed altered expression levels in these ES cells. We noted three groups of genes: genes showing more than twofold downregulation (*Cdc25a*, *Cdc42*, *Cdh1/E-cadherin*, and *Ezh2*), genes showing more than twofold upregulation (*Nfyc*, *Mapk6*, *Sox13*, and *Nr5a2*), and genes showing small or no changes (*Conf*, *Idb2*, *Miz1*, *Mta3*, and *Oct3/4/Pou5f1*). Although the expression levels of *Fancg* and *Zfp42/Rex1* showed less than twofold changes, they may also be affected by the loss of *Esg1* expression. The downregulation of *Ezh2*, a transcriptional repressor, and the upregulation of *Nfyc* and *Nr5a2*, transcriptional activators, might suggest an indirect role for ESG1 in transcriptional suppression. Because *Cdc25a* degradation by ectopic *Chk2* expression caused the appearance of G1 phase, which is normally absent in ES cells (Burdon *et al.* 2002; Hong & Stambrook 2004), the downregulation of *Cdc25a* seems to be consistent with the prolonged doubling time of $Esg1^{-/-}$ ES cells compared to that of intact ES cells (Table 1), although the variations of the doubling time between two $Esg1^{-/-}$ ES clones and between two $Esg1^{+/-}$ ES clones were relatively large.

We also noticed that there were considerable variations of each gene expression level among ES cell lines of each genetic background (intact $Esg1^{+/+}$, $Esg1^{+/-}$ or $Esg1^{-/-}$; Table 2, Fig. 3D). The variations in expression of prospective *Esg1* targets among *Esg1*-manipulated lines were 4–20-fold higher than the variations among the biological replications of $Esg1^{+/+}$ ES cells. These results seem to hint some differences in the gene expression patterns among *Esg1*-manipulated ES cells, which may be consistent with the variations of the cell doubling time between two $Esg1^{-/-}$ ES clones and between two $Esg1^{+/-}$ ES clones.

Discussion

It is now firmly established that the expression of *Esg1*, both RNA and protein, is restricted to cells in the pluripotent cycle (this work; see also Western *et al.* 2005), where *Oct3/4* and *Nanog* had previously been the only genes that show this idiosyncratic expression pattern. *Esg1* seems to be a downstream of *Oct3/4*, because the forced repression of *Oct3/4* in mouse ES cells immediately downregulates the expression of *Esg1* (Tanaka *et al.* 2002). Therefore, *Esg1* will be an excellent marker for cells in pluripotent cycle and undifferentiated ES cells.

Table 1. Proliferation rates of $Esg1^{+/-}$ and $Esg1^{-/-}$ cells

	Average doubling time (mean ± SE) ($n = 4$)	*P*-value (compared to 129.3)
129.3 intact $Esg1^{+/+}$	10.7 ± 0.4	
$Esg1^{+/-}$ clone #1	15.9 ± 0.9	0.001
$Esg1^{-/-}$ clone #33	19.5 ± 1.0	<0.001
$Esg1^{-/-}$ clone #2	11.8 ± 1.3	0.474
$Esg1^{-/-}$ clone #27	19.2 ± 1.1	0.001

P-values were calculated by the *T*-test, paired for single means.

It has been suggested that ESG1 is an RNA-binding protein, because it contains 118 amino acids with a putative heterogeneous nuclear RNP K-homology (KH)-domain, which is conserved among the RNA-binding proteins such as the Fragile X mental retardation gene *FMR1*, *Nova1*, *Sam68*, *gld-1*, and quakinguiable protein (*Qki*) (Siomi & Dreyfuss 1997; Adinolfi *et al.* 1999; Perrone-Bizzozero & Bolognani 2002). We now provide more evidence to support this notion. Like other RNA-binding proteins, ESG1 seems to form a complex with a broad range of RNA targets (~1000) in ES cells. The mRNA targets of ESG1 include those encoding proteins that regulate the cell division cycle, chromatin remodeling, and gene transcription. Because the KH-motif was often found in multiple copies and followed by other conserved motifs in the most of the proteins in the family (Adinolfi *et al.* 1999), ESG1 containing only one KH-motif might recruit other proteins to be functional, so that the functional protein complex(es) would regulate the metabolism of its RNA targets. It is very interesting to note that *Esg1* mRNA itself is included in this list, indicating that ESG1 binds to its own mRNA and possibly regulates its own expression.

RNA-binding proteins play important roles in germ cell development. For example, in *Caenorhabditis elegans*, two RNA-binding proteins with KH-motifs, GLD-1 and MEX-3, are localized in the germline determinant P-granules and play important roles in germ cell establishment (Draper *et al.* 1996; Schisa *et al.* 2001). It is thus tempting to speculate that ESG1 also plays a critical role in pluripotent embryonic cells and germ cells. However, $Esg1^{-/-}$ ES cells and $Esg1^{-/-}$ mice did not show any obvious abnormality in embryonic development, fertility, the derivation of ES cells from blastocysts, the growth and differentiation of ES cells (Amano *et al.* in press), although it still remains unclear that results obtained by utilizing $Esg1^{-/-}$ ES cells were unique due to the selection of cells adopted to the microenvironment missing functional ESG1. In fact, a prolonged doubling time of $Esg1^{-/-}$ ES clones was observed, although the clone-to-clone variation was too large to make this argument conclusive. Such clone-to-clone variations

Gene	Genotype	*df1*	*df2*	MS	*F*	*P-value*
Cdc25a	+/-	1	6	0.48	0.37	0.564
	-/-	2	9	0.04	82.49	<0.001*
Cdc42	+/-	1	6	0.31	5.01	0.067
	-/-	2	9	0.02	8.25	0.009*
Ccnf	+/-	1	6	0.09	274.03	<0.001*
	-/-	2	9	0.09	184.45	<0.001*
Cdh1	+/-	1	6	0.51	2.34	0.177
	-/-	2	9	0.00	58.26	<0.001*
Ezh2	+/-	1	6	0.15	1.46	0.273
	-/-	2	9	0.06	140.70	<0.001*
FancG	+/-	1	6	0.01	7.09	0.037*
	-/-	2	9	0.17	2.94	0.104
Nyc	+/-	1	6	0.03	3.03	0.132
	-/-	2	9	0.50	5.32	0.030*
Nr5a	+/-	1	6	0.00	1.44	0.275
	-/-	2	9	0.01	574.54	<0.001*
Zfp42	+/-	1	6	0.09	95.95	<0.001*
	-/-	2	9	0.19	3466.60	<0.001*
Oct3/4	+/-	1	6	0.03	146.58	<0.001*
	-/-	2	9	0.95	10.52	0.004*
CcnD3	+/-	1	6	0.00	18.85	0.005*
	-/-	2	9	0.00	8.54	0.008*
Idb2	+/-	1	6	3.95	1438.02	<0.001*
	-/-	2	9	2.44	378.00	<0.001*
Mapk6	+/-	1	6	0.00	20.74	0.004*
	-/-	2	9	0.21026	20.00	<0.001*
Miz1	+/-	1	6	0.00146	213.03	<0.001*
	-/-	2	9	0.01088	437.15	<0.001*
Mta3	+/-	1	6	5E-05	0.15	0.708
	-/-	2	9	0.30438	6409.26	<0.001*
Sin3a	+/-	1	6	2.3E-09	12.90	0.011*
	-/-	2	9	3.2E-07	13 4898.46	<0.001*
Sox13	+/-	1	6	8.8E-06	748.29	<0.001*
	-/-	2	9	1.8E-05	4989.88	<0.001*

Statistical significance of the difference in expression levels of each gene indicated on the left among clones with the same genotype (see Figure 3D) were calculated by the single-factor ANOVA. *Statistically significant. df, degrees of freedom; ES, embryonic stem; MS, mean square error; F, Fisher statistics.

Table 2. Clonal variations of gene expression levels within two *Esg1*$^{+/-}$ ES clones and within three *Esg1*$^{+/+}$ ES clones

can be explained by the intrinsically stochastic nature of gene expression regulation (Ko 1992; Kaern *et al.* 2005; Raser & O'Shea 2005).

The subtle phenotype of *Esg1*$^{-/-}$ ES cells and *Esg1*$^{+/-}$ mice were not entirely unexpected, considering the similar subtle phenotypes observed in the targeted mutagenesis of other RNA-binding proteins. For example, KH-motif proteins *Vera/Vg1-binding protein* in *Xenopus*, and its homologue *Zipcode-binding protein 1* (*ZBP1*) in chick translocate their target mRNA, *Vg1* and *β-actin*, to specific sites in the cytoplasm (Deshler *et al.* 1998). The mouse homologue of *ZBP1*, *Insulin-like growth factor 2 binding protein 1* (*Imp1*) has been reported to be expressed widely in oocytes, zygotes, and E12.5 embryos, but gene-trap mutagenesis of the gene showed postnatal lethality only in 50% of homozygous mutant mice (Hansen *et al.* 2004). Similarly, homozygous mutant mice of another KH-motif protein, *Sam68*, showed defects only in age-related bone metabolism (Richard *et al.* 2005), even though *Sam68* protein was widely localized in E14.5 and 16.5 embryos. These RNA-binding proteins may thus function, not as a determinant, but as a modulator of cellular functions through their binding to a large number of target mRNA species (for example, Lopez de Silanes *et al.* 2004). It remains to be formally investigated whether *Esg1* influences the stability of target RNA, their subcellular localization, and/or their translation rate.

In conclusion, pluripotent cycle-specific *Esg1* is most likely an RNA-binding protein with broad spectrum of target RNA. Although *Esg1* is dispensable in ES cells and mice according to gene disruption studies, whether *Esg1* has an important function in cells under stress, during aging, or in other pathological conditions remains to be investigated.

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To help facilitate research with hES cells we describe in detail the protocols used in our laboratory for the derivation and maintenance of hES cell lines (Cowan *et al.*, 2004; Klimanskaya and McMahon, 2004; http://mcb.harvard.edu/melton/hues). In addition, we briefly discuss alternative approaches to the maintenance of hES cells. Thus, this chapter provides a starting point for researchers interested in establishing and working with hES cell lines.

Derivation of hES Cell Lines

Since the initial derivation of human ES cell lines by Thomson *et al.* (1998), several additional hES cell lines have been established and characterized (Table I; www.stemcells.nih.gov/registry/index.asp). We reported previously the derivation and maintenance of 17 new hES cell lines that can be maintained in culture by enzymatic dissociation with trypsin (Cowan, 2004). Our complete protocol has been described previously in detail (Klimanskaya and McMahon, 2004). The general utility and success of our approach have been validated by the transfer of this technique to several researchers and their subsequent derivation of new hES cell lines (Melton and Eggan, unpublished data). This chapter presents our most concise and current protocol for the derivation of hES cell lines.

Planning and Considerations

In our experience, hES cell derivation can be rather time-consuming and demanding. Until the isolated cells are frozen and thawed, they must be continually passaged and maintained. On average, one can expect 3 to 6 weeks of uninterrupted culture from the point of initiating an attempt to isolate hES cells from blastocyst embryos. Before deriving any new hES cell lines, we recommend that all of the reagents necessary for culture and derivation of the cells be obtained and, if possible, tested by routine culture of preexisting hES cell lines. Our standard derivation protocol makes use of mouse embryonic fibroblast cells as a feeder layer, and we also recommend the isolation and testing of these cells before attempting to isolate new hES cell lines. Finally, our protocol is designed to derive hES cells from blastocyst stage embryos, and while we have derived several cell lines from embryos frozen at early cleavage stages, they are always first cultured until they mature into blastocysts. In the following sections we will attempt to walk the reader through a stepwise protocol for deriving hES cell lines and, when necessary, to provide specifics details as to the suppliers of certain essential reagents.

TABLE I
PUBLISHED HUMAN EMBRYONIC STEM CELL DERIVATIONS[a]

No. of established lines	No. of plated embryos	Karyotype		Feeder source	Isolation of ICM	Medium for isolation of ICM	References
		46,XX	46,XY				
5	14	3	2	irrad-MEF	IS	20% FBS	Thomson *et al.* (1998)
2	4	2	0	mitoC-MEF	IS	20% FBS+LIF	Reubinoff *et al.* (2000)
1	1	0	1	mitoC-HFM	IS	20% HS+ITS	Richards *et al.* (2002)
1	4	1	0	irrad-HFF	IS	20% FBS+LIF	Hovatta *et al.* (2003)
3	30	1	2	mitoC-STO	IS	20% FBS+LIF	Park *et al.* (2003)
2	19	0	2	inact-MEF	IS	20% FBS+LIF+bFGF	Mitalipova *et al.* (2003)
6	N/A[b]	1[c]	5[d]	mitoC-MEF	IS/WB	20% SR+bFGF+HA	Heins *et al.* (2004)
1	1	1	0	mitoC-MEF	WB	20% FBS+LIF	Baharvand *et al.* (2004)
17	97	8	9	irrad-MEF	IS	8% SR+8% plasmanate+ LIF+bFGF	Cowan *et al.* (2004)
1	9	1	0	inact-MEF	WB	20% FBS	Suss-Toby *et al.* (2004)
1	7	1	0	irrad-MEF	IS	10% FBS	Stojkovic *et al.* (2004)
9	20	4	5	mitoC-STO	IS	20% FBS+LIF+bFGF	Park *et al.* (2004)
9	19	6	3	mitoC-MEF	IS	20% SR	Kim *et al.* (2005)
2	10	1	1	irrad-HFF	IS	20% SR+bFGF	Inzunza *et al.* (2005)
2	16	2	0	HPF	IS	20% SR+bFGF	Simon *et al.* (2005)

2	111	1	1	irrad-HPF	WB	20% SR+bFGF	Genbacev *et al.* (2005)
3	7	3	0	mitoC-HUE	IS	20% SR+bFGF	Lee *et al.* (2005)
3	10	1	2	mitoC-STO	IS/WB	20% SR+bFGF	Oh *et al.* (2005)
1	5	1	0	free[e]	IS	8% SR+8% plasmanate+ LIF+bFGF	Klimanskaya *et al.* (2005)
2	19	1	1	mitoC-MEF	IS	20% FBS+bFGF	Chen *et al.* (2005)
2	55	0	2	irrad-MEF	IS	20% FBS+LIF+bFGF	Mateizel *et al.* (2005)
3	14	2	1	irrad-MEF	IS	20% SR+bFGF	Mateizel *et al.* (2005)
4	14	2	2	irrad-MEF	IS	20% SR+bFGF	Hong-mei and Gui-an (2006)
1	33	1	0	inact-MEF	WB	20% FBS+bFGF	Sun *et al.* (2006)
2	5	0	2[f]	free[g]	IS	TeSR1[h]	Ludwig *et al.* (2006)

[a] irrad, irradiated; mitoC, mitomycin C; MEF, mouse embryonic feeders; HFM, human fetal muscle; HFF, human foreskin fibroblasts; STO, STO cells; HPF, human placental fibroblasts; HUE, human uterine endometrial cells; IS, immunosurgery; WB, whole blastocyst; FBS, fetal bovine serum, HS, human serum; SR, Serum Replacement; ITS, insulin transferring selenium; HA, hyaluronic acid.

[b] Information not available from published sources.

[c] XX karyotype with a trisomy 13.

[d] One line was a triploid, 69,XXY.

[e] Mouse extracellular matrix coated.

[f] One line was a 47,XXY.

[g] Human extracellular matrix coated.

[h] TeSR1was highly defined medium, which was composed of a DMEM/F12 base supplemented with human serum albumin, vitamins, antioxidants, trace minerals, specific lipids, and growth factors.

Preparation of Mouse Embryo Fibroblasts (MEFs)

We use primary MEF cells, which have been mitotically inactivated by γ-irradiation, for derivation and propagation of hES cells. MEFs are harvested from 12.5-day postcoitum (dpc) fetuses of ICR mice (Cowan *et al.*, 2004). The following reagents are required to follow our protocol for preparing MEFs.

Sterile phosphate-buffered saline (PBS), pH 7.2
MEF medium (90% Dulbecco's modified Eagle's medium [DMEM], 10% fetal bovine serum [FBS], 50 units/ml penicillin, and 50 μg/ml streptomycin)
0.25% trypsin
0.1% gelatin (made by dissolving 1 g of gelatin in 1000 ml of Milli-Q quality water, followed by sterile filtering)
Freezing medium (90% FBS, 10% dimethyl sulfoxide [DMSO])
10- and 15-cm tissue culture dishes
Sterile single-edged razor blade

Dissection and Primary Culture of MEFs

Prior to dissecting the mouse embryos, several 15-cm tissue culture plates (seven to eight plates per pregnant ICR female) should be coated with 0.1% gelatin. We typically cover the plates with a minimal amount of the gelatin solution (5 to 7 ml) and incubate them for 20 min at 37° with 5% CO_2. Using a microscope placed in a laminar flow hood, 12.5-dpc embryos are dissected into a 10-cm Petri dish containing sterile PBS solution. The embryos are then stripped of any maternal or extraembryonic tissues and eviscerated. Eviscerated embryos are transferred to a 15-cm dish and, using a sterile blade, minced. Ten milliliters of warm 0.25% trypsin is added per 10 to 14 minced embryos and collected in a 50-ml conical tube. The embryos are homogenized further by trituration (pipetting up and down) until no large pieces remain. This partially dissociated mixture is then incubated at 37° for 1 min followed by further trituration (pipetting 5 to 10 more times). Forty milliliters of prewarmed MEF medium is added to the dissociated embryos and the mixture is centrifuged for 10 min at 500 to 600*g* at room temperature. Aspirate media and then resuspend the pelleted cells with 30 ml prewarmed MEF medium. Plating density is 1.5 to 2 embryos per 15-cm gelatin-coated plate. The final volume of medium on each plate should be 20 ml. The primary MEFs are incubated at 37° with 5% CO_2 until confluent (typically 5 to 6 days). MEFs are expanded once after the initial plating (1:3 to 1:5 split) and then frozen (passage 1). Freeze MEFs in freezing medium (90% FBS and 10% DMSO) at a rate of −1°/min and store at −80° or in liquid nitrogen.

γ-Irradiation and Plating

Thawed MEFs are only passaged once (passage 2) for expansion purposes prior to γ-irradiation. MEFs are trypsinized and resuspended in a volume of MEF medium that will be accommodated by the γ-irradiator. Irradiate the MEFs for 25 min at 247.3 rad/min for a total exposure of 6182.5 rad. After irradiation, spin cells in MEF medium for 5 min at 500 to 600*g*. To ensure a confluent monolayer, plate MEFs at a concentration of approximately 50,000 cells/cm^2. If there is no immediate need for mitotically inactivated MEFs, they can be frozen at a concentration of 4×10^6 to 1.2×10^7 cells/vial. MEFs feeder layers should be prepared and used within 3 days.

Preparing hES Derivation Medium

During the isolation and early stages of ES cell cultivation, hES derivation medium is used, which consists of 75% knockout DMEM (Invitrogen GIBCO), 10% KO-Serum Replacement (Invitrogen GIBCO), 10% plasmanate (Bayer), 5% fetal bovine serum (Hyclone), 2 m*M* Glutamax-I (Invitrogen GIBCO), 1% nonessential amino acids (Invitrogen GIBCO), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen GIBCO), 0.055 m*M* β-mercaptoethanol (Invitrogen GIBCO), 12 ng/ml recombinant hLIF (Chemicon International), and 5 ng/ml bFGF (Invitrogen GIBCO). The medium is sterilized by 0.22-μm filtration. Screening of FBS, plasmanate, and Serum Replacement should be done and is described elsewhere (Klimanskaya and McMahon, 2004).

Isolation of Inner Cell Mass

Fresh or frozen-thawed human embryos are cultured to the blastocyst stage in sequential media, G1.2 and G2.2 (Gardner *et al.*, 1998). We have derived several new human ES cell lines at relatively higher efficiency from blastocysts cultured in modified KSOM media. Blastocysts are treated with acid tyrodes (Specialty Media) for 30 to 90 s to dissolve the zona pellucida. When the zona pellucida starts to dissolve, remove the embryo and wash it three times in fresh hES derivation medium. The zona-stripped embryos are then cultured in hES derivation medium at 37° with 5% CO_2 until immunosurgical isolation of the ICM. The process of immunosurgery includes several stages and is performed essentially as described by Solter and Knowles (1975). Initially, the embryo is incubated for approximately 30 min in rabbit antihuman RBC antibodies (resuspended as per manufacturer's instructions, aliquoted, and stored at −80°, freshly diluted 1:10 in derivation medium, Inter Cell Technologies). Penetration of the antibodies into the blastocyst is prevented because of cell–cell connections within the outer layer of the

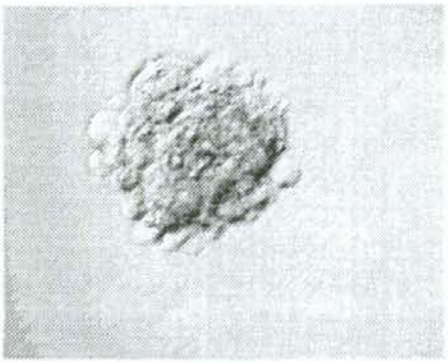

FIG. 1. "Bubbling" of trophoblast cells. Blastocyst after exposure to guinea pig sera complement is lysed and stop the incubation followed by removing the lyses trophoblast cells.

trophoblasts, leaving the ICM intact. After rinsing off any antibody residue (at least three washes with hES derivation media), the blastocyst is transferred into a guinea pig sera complement (resuspended as per manufacturer's instructions, aliquoted, and stored at −80°, freshly diluted 1:10 in derivation medium, Sigma), diluted in hES derivation medium, and incubated until cell lysis is notable, indicated by an apparent "bubbling" of the trophoblast cells (Fig. 1). Following selective removal of the trophectoderm cells by gentle mouth pipetting of the embryo in and out of a glass capillary, the intact ICM is cultured on MEF feeders plated on gelatin (Sigma)-coated tissue culture plates at a density of approximately 50,000 cells/cm^2. After 2 days add a few fresh drops of hES derivation medium and then every other day change one-half the total medium (e.g., for 500 μl total medium and then remove 250 μl of medium and add 250 μl of fresh medium to a final volume of 500 μl).

Dispersion of Inner Cell Mass

Six to 10 days after the initial plating, ICM outgrowths require mechanical dissociation. Two to three pieces are cut from the initial outgrowth using a narrow glass capillary and are left in the same well or moved to a new well (Fig. 2). When doing the initial dispersion, a part of the original colony should be left untouched as a backup, especially if the picked pieces are transferred into a new well. At this stage, it is better to concentrate on expanding the number of hES cell colonies versus freezing or proceeding to any downstream experiments. When the colonies are growing steadily, FBS is omitted from the culture media. Usually, mechanical passaging needs to be done every 5 to 6 days, but several larger colonies may need to be dispersed daily.

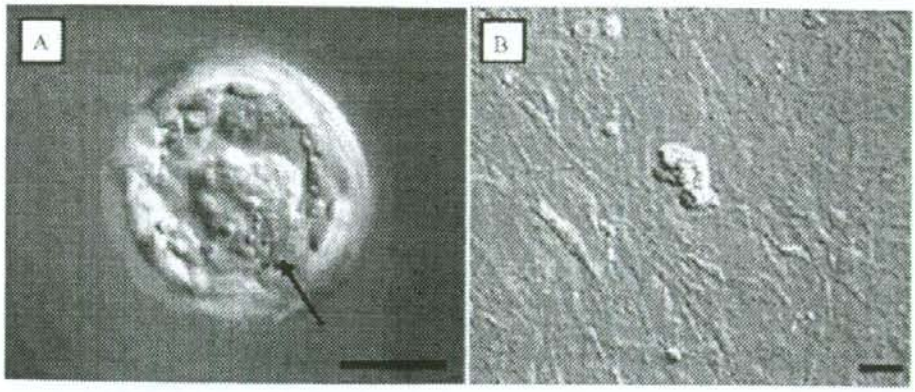

FIG. 2. Blastocyst and ICM outgrowth. (A) Cultured blastocyst is grade 4AA. Arrow indicates ICM. (B) Isolated ICM from the blastocyst (A) is just grown ICM at day 4 after plating on mitotically inactivated MEFs. Black bar: 50 μm.

Maintenance of hES Cells

Variability among human ES cell lines has been reported by several groups, including differences in growth characteristics, differentiation potential, karyotype, and gene expression pattern. In part, these differences might reflect the genetic heterogeneity of hES cell lines derived, as they are from a genetically diverse, outbred population (Abeyta *et al.*, 2004; Bhattacharya *et al.*, 2004). Further confounding researchers is the fact that human ES cell cultures are often heterogeneous because they contain both undifferentiated stem cells and spontaneously arising differentiated derivatives. While no single uniform protocol exists for the maintenance of hES cells in culture that adequately addresses all researchers' concerns, we will attempt to present an overview of the techniques currently used by a number of laboratories around the world. Again, we describe in detail our method for maintaining undifferentiated hES cell growth in culture and briefly review several alternative protocols.

Enzymatic Dissociation with Trypsin

Human ES cell colonies are passaged by mechanical dissociation until there are sufficient colonies (50 to 100 average-sized colonies) or cells (usually 1×10^5 cells) to passage enzymatically. Thereafter, hES cells are propagated by enzymatic dissociation with 0.05% trypsin/EDTA (Invitrogen GIBCO). During the first three passages with trypsin, it is a good idea to keep a backup well of mechanically passaged cells. A mechanical backup should always be maintained until the cells are frozen. Subconfluent cultures are generally split at a 1:3 ratio (i.e., one culture well is split into three new culture wells). It is important to split colonies prior to excessive differentiation.

Materials

For the routine culture of hES cell by enzymatic dissociation with trypsin we recommend the following media and reagents.

hES medium (80% knockout DMEM, 10% KO-Serum Replacement, 10% plasmanate, 2 m*M* Glutamax-I, 1% nonessential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.055 m*M* β-mercaptoethanol, and 5 ng/ml bFGF

Trypsin 0.05%

Sterile PBS, pH 7.2.

Trypsinization

1. Warm hES medium and trypsin in a 37° water bath and keep them warm until ready for use.

2. Place MEF plate from incubator in the hood and aspirate off the medium from the well followed by 1 ml prewarmed hES medium. Set the plate aside in the hood.

3. Carefully aspirate the hES medium from the culture to be split. Gently rinse the cells with a sufficient volume of PBS to completely cover the bottom of the culture dish (e.g., 5 ml for a 10-cm dish).

4. Aspirate the PBS and add a small volume of trypsin (usually 0.3 ml for a 35-mm well or 2 ml for a 10-cm dish) to the cells. Incubate in the hood at room temperature, frequently checking the cells under the microscope. MEFs surrounding the colonies should begin to retract (Fig. 3). When the MEFs are sufficiently shrunk and the borders of the colonies are roughly rounded up, add 10 volumes of prewarmed hES medium to the trypsinized colonies. Gently pipette up and down five to seven times until the MEF monolayer has completely detached. Extensive pipetting should be avoided.

5. Aliquot the hES cell solution dropwise, making sure to distribute the drops evenly about the well. Without shaking the plate, carefully return to the cells to a 37° incubator overnight to let the colonies seed.

The time in trypsin required for the cells to detach varies depending on the hES cell density, age of MEF monolayer, etc. We recommend checking the appearance of the hES culture under a stereomicroscope and determining the best incubation time for each well empirically.

Freezing hES Cells

1. Trypsinize the cells; see trypsinization section. Centrifuge the cells at 600*g* in 10 volumes of hES culture media.

2. Resuspend the pellet in cold freezing medium, which consists of 90% FBS and 10% DMSO.

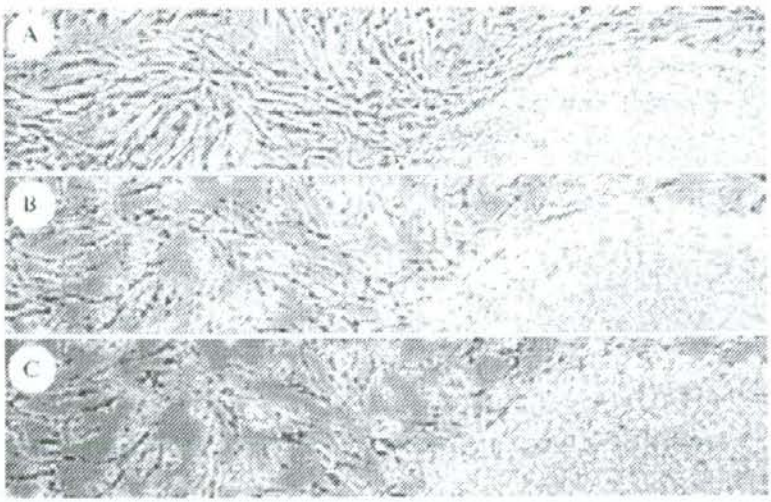

FIG. 3. Time-lapse series of photographs showing dissociation of hES cells and MEF feeder layer with trypsin. (A) Prior to addition of trypsin. (B) Approximately 30 s after addition of trypsin. (C) Approximately 60 s after addition of trypsin. Trypsinization should be stopped when cells appear as in C.

3. Aliquot the cell suspension into prechilled freezing vials and sandwich the vials between two Styrofoam racks, taping to prevent them from separating. Transfer to a −80° freezer overnight. Cryovials should be placed in liquid nitrogen for long-term storage.

Thawing hES Cells

Ensure that the MEF plate prepared is confluent and in good condition before thawing hES cells. Prewarm hES medium to 37°. Aliquot 10 ml hES medium into a sterile and labeled 15-ml conical tube for each cell line.

All procedures should be done quickly.

1. Thaw the vial in a 37° water bath. (Do not overthaw; the vial should be removed from the water bath with a small ice crystal still remaining.) It should take about 45 to 60 s before the cells are 80% thawed.
2. Bring the tube to a laminar flow hood; spray down with 70% isopropanol. Gently transfer cells to the 10 ml of prewarmed medium.
3. Centrifuge the 15-ml conical tube at 500 to 600*g* for 5 min.
4. Remove preplated MEFs from incubator to the hood. Aspirate off the MEF medium and aliquot prewarmed hES medium into each well of the plate, being careful not to disturb the attached MEFs.
5. After the spin is complete, carefully remove the medium without disturbing the pellet.

6. Gently resuspend the pellet in a small volume of prewarmed hES medium.

7. Transfer the hES cell solution, in a dropwise manner, to a prepared MEF plate well that already contains hES medium. Carefully return the plate to avoid swirling to a 37° incubator overnight to allow the hES cells to seed the MEFs.

8. The colonies usually begin to appear in 3 to 4 days and can be ready for splitting in 5 to 10 days.

Alternative Methods

Several alternative methods exist for the culture of hES cells, but few have been examined rigorously over a long period of time. We will attempt to summarize some of the more common alternative methods for maintaining hES cells in culture. For detailed protocols, we advise referring to the primary literature. In addition, several alternatives, such as feeder and serum-free culturing of hES cells, are described elsewhere in this volume.

Dissociation with Collagenase or Dispase

Quite possibly the most widespread method for maintaining hES cells in culture depends on their dissociation with either collagenase or dispase. For a detailed protocol, please see http://www.geron.com/PDF/scprotocols.pdf. The reported advantages of culture with these enzymes are reduced cell death and perhaps greater karyotypic stability. The disadvantages of enzymatic dissociation with collagenase or dispase include the inability to accurately assess cell number and the failure to generate definitive single cell clones.

Culture with Human Feeder Cells

Mouse embryonic fibroblast cells have generally been used as feeder layers to support the unlimited growth of hES cells, but the use of animal feeder cells is associated with risks such as pathogen transmission and viral infection (Amit *et al.*, 2003, 2004; Richards *et al.*, 2002; Rosler *et al.*, 2004). Martin *et al* (2005) reported that hES cells could incorporate foreign sugars into the glycoproteins on the cell surface. They also showed that an immune reaction could occur following exposure of the cells to serum from adults with high level of the antibody. These reports and other concerns have prompted many researchers to seek alternatives to mouse feeder layers.

Several groups have reported that feeder layers composed of cells originating from human fetal and adult tissues support unlimited proliferation of hES cells without differentiation. The cell types used include human

fetal skin fibroblasts, human muscle cells, adult fallopian tubal epithelial cells (Richards *et al.*, 2002), adult marrow cells (Cheng *et al.*, 2003), foreskin fibroblasts (Amit *et al.*, 2003; Hovatta *et al.*, 2003), human uterine endometrium cells, and breast parenchyma cell abortus fetus fibroblasts (Lee *et al.*, 2004). In perhaps the most comprehensive study, Richards *et al.* (2003) reported on the evaluation of various human adult, fetal, and neonatal tissues as feeder cells for supporting the growth of hES cells. In addition, feeder cells derived from hES cells can be used as an autogenic feeder system that efficiently supports the growth and maintenance of pluripotency of hES cells (Stojkovic *et al.*, 2005; Yoo *et al.*, 2005).

Conclusion

Human ES cells are viewed by many as a novel and unlimited source of cells and tissues for transplantation for the treatment of a broad spectrum of diseases (reviewed by Keller, 2005). Moreover, human ES cells represent an unprecedented system suitable for the identification of new molecular targets and the development of novel drugs, which can be tested *in vitro* or used to predict or anticipate potential toxicity in humans. Finally, human ES cells can yield insight into the developmental events that occur during human embryogenesis, which are, for ethical reasons, nearly impossible to study in the intact embryo (reviewed by Dvash and Benvenisty, 2004).

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