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H．知的財産権の出願・登録状況

特記すべき事なし

分担研究報告書

男性不妊症感受性遺伝子同定のための連鎖不平衡マッピング

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研究要旨：同定された男性不妊症遺伝子座から、効率よく疾患感受性遺伝子を同定するにはヒトゲノムにおける連鎖不平衡を応用する方法が最も効率的であると考えられる。そのためにはヒトゲノムにおける連鎖不平衡の成り立ち、およびヒトゲノムの多様性を理解することが不可欠である。我々は男性不妊症感受性の候補遺伝子である ADP-ribosyltransferase (*ART*) 遺伝子における連鎖不平衡、ゲノムの多様性に関する理解を深めるために全世界の 13 集団 (アフリカ 3 集団、ユーラシア 6 集団、東アジア 4 集団) から収集した 585 人の*ART* 遺伝領域の塩基配列を詳細に比較した。特に アフリカンアメリカン、コーカサス、日本人集団に共通する男性不妊症で有意な 18 個の遺伝子多型による連鎖不平衡の解析を行った。アフリカンアメリカン、コーカサス集団に比べ、日本人集団では連鎖不平衡が保たれていることが示された。疾患遺伝子の同定には連鎖不平衡のブロック構造解析不可欠であると考えられた。

一方、各々の大陸集団に特異的な塩基配列の変化も多数存在することが確認され、現在の common variant、common haplotype を中心とした common disease 感受性遺伝子の同定戦略にも今後留意する必要があると考えられる

A．研究目的

不妊症の原因究明に関し、女性側要因のみでなく男性側要因へのアプローチも重要である。もとより、不妊の原因として男性要因が 25%程度を占めるといわれている上、最近では環境ホルモンの影響もあり男性不妊症の増加が危惧されている。本研究では男性不妊症に的を絞り、男性不妊症患者から得た精巣組織での遺伝子発現をマイクロアレイ法にて検討し、遺伝子発現プロファイルからの病態解析を試みた。さらに、ケースコントロール・アソシエーションスタディーにより、遺伝要因となる候補遺伝子を絞り込んだ。

この遺伝子と男性不妊症の関連を効率良く調べるために、連鎖不平衡を応用することが最も効率的であると考えられる。

連鎖不平衡とは 2 つ (以上) の変異が連鎖している状態であり、異なった座位の SNP が強い連鎖不平衡にあれば患者・対照者関連試験を行った場合に同じような疾患との関連が認められる。つまり、あるゲノムの領域のひとつの SNP とその関連を調べれば、その領域と連鎖不平衡の関係にある複数の SNP と疾患の関連が明らかになることになり、連鎖不平衡を利用すれば効率よく SNP と疾患との関連を調べることができる。この理論を応用したのが連鎖不平衡マッピング法である。しかし、ヒトの遺伝子における連鎖不平衡の成り立ちについては詳細な検討がほとんどなされておらず、理論が先行している点は否めない。また、連鎖不平衡の成り立ちは人種、民族により大きく異なることが予想される。

本研究では疾患感受性遺伝子の解明に不可欠な連鎖不平衡マッピング法を、より実践的なマッピング法として確立するためにヒトの遺伝子における連鎖不平衡の成り立ち、ヒトゲノムの多様性を明らかにしようとするものである。そのモデル研究として、今

回は *ART* 遺伝子の連鎖不平衡の解明を行うことを目的とした。

B．研究方法

1)対象　全世界の 13 集団（アフリカ 3 集団、ユーラシア 6 集団、東アジア 4 集団）から収集した 585 人の ゲノム DNA。

2) ヒト *ART* 遺伝子を含む 48kb にわたる領域の SNP のタイピング

データベース上のヒトのこの領域の塩基配列を基に、必要な PCR およびシークエンスプライマーを作成した。ゲノム DNA を PCR で増幅し、自動シークエンス解析装置 (ABI PRISM 3700) を用いたダイレクトシークエンス法、または、TaqMan® SNP Genotyping Assays にて、SNP のタイピングを行った。

3) ヒト *ART* 遺伝子領域における連鎖不平衡の検討

遺伝子多型のタイピングデータを基に各遺伝子間での連鎖不平衡の解析を行う。SNP 間の連鎖不平衡は、D (Lewontin and Kojima, 1960)、D' (D/D_{max}) (Leowntin 1964)、r^2 ($D^2/p_1(1- p_1)p_{2(}(1-p_2)$)、およびカイ検定（$\chi^2$値、p 値）にて評価した。

（倫理面への配慮）ヒト DNA サンプルについては、購入元 (Coriell Institute for Medical Research) に、医学部長の承認を得たことを証明する許諾書を提出済み。

C．研究結果

全世界の全世界の 13 集団（アフリカ 3 集団、ユーラシア 6 集団、東アジア 4 集団）から収集した 585 人の ゲノム DNA の Exon に存在する 3 SNP のハプロタイプ頻度について系統解析を行ったところ、各集団でグループを形成することが確認された。さらに、アフリカンアメリカン、コーカサス、日本人集団に共通する男性不妊症で有意な 18 個の遺伝子多型による連鎖不平衡の解析を行った。アフリカンアメリカン、コーカサス集団に比べ、日本人集団では連鎖不平衡が保たれていることが示された。

各々の大陸集団で特異的な 遺伝子頻度、ハプロタイプ頻度を示すことが確認された。

D．考察

連鎖不平衡の強さは集団サンプルによって異なり、疾患感受性遺伝子の同定には日本人を対象とした詳細な連鎖不平衡の検討が不可欠であると考えられる。また、各々の集団に特異的な遺伝子頻度の変化も多数存在することが確認され現在の Common variant, common haplotype を中心とした common disease 感受性遺伝子の同定戦略にも今後留意する必要があると考えられる。

E．結論

男性不妊症の疾患感受性遺伝子の同定に向け、*ART* 遺伝子領域における詳細な連鎖不平衡の検討を行った。今後、この見地をもとに遺伝子多型と男性不妊症の関連を効率よく進めていくことが可能であると考えられる。

F．健康危険情報

特になし。

G．研究発表

なし

H．知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

Ⅲ　研究成果に関する一欄表

別紙５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ　研究成果の刊行物・別冊

Develop. Growth Differ. (2006) **48**, 381–390 doi: 10.1111/j.1440-169x.2006.00875.x

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

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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*, *Nlyc* 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*

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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

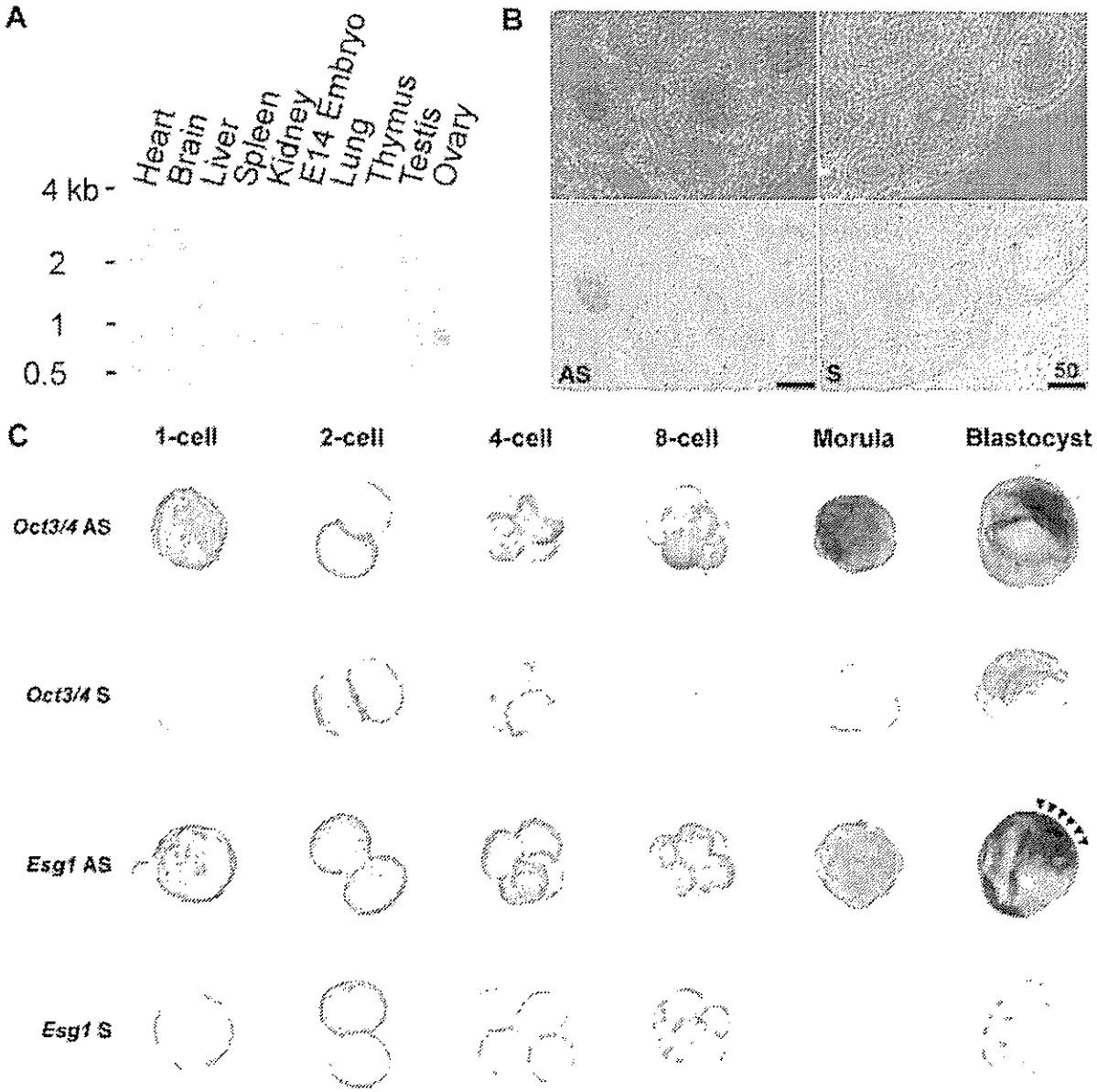

Fig. 1. Expression patterns and localization of *Esg1* transcript. (A) Northern hybridization analysis showed a ~0.7 kb transcript of *Esg1* in adult ovary and to a lesser extent, in testis. (B) *In situ* hybridization on the section of adult ovary. Phase contrast images are shown on the top. AS, antisense 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

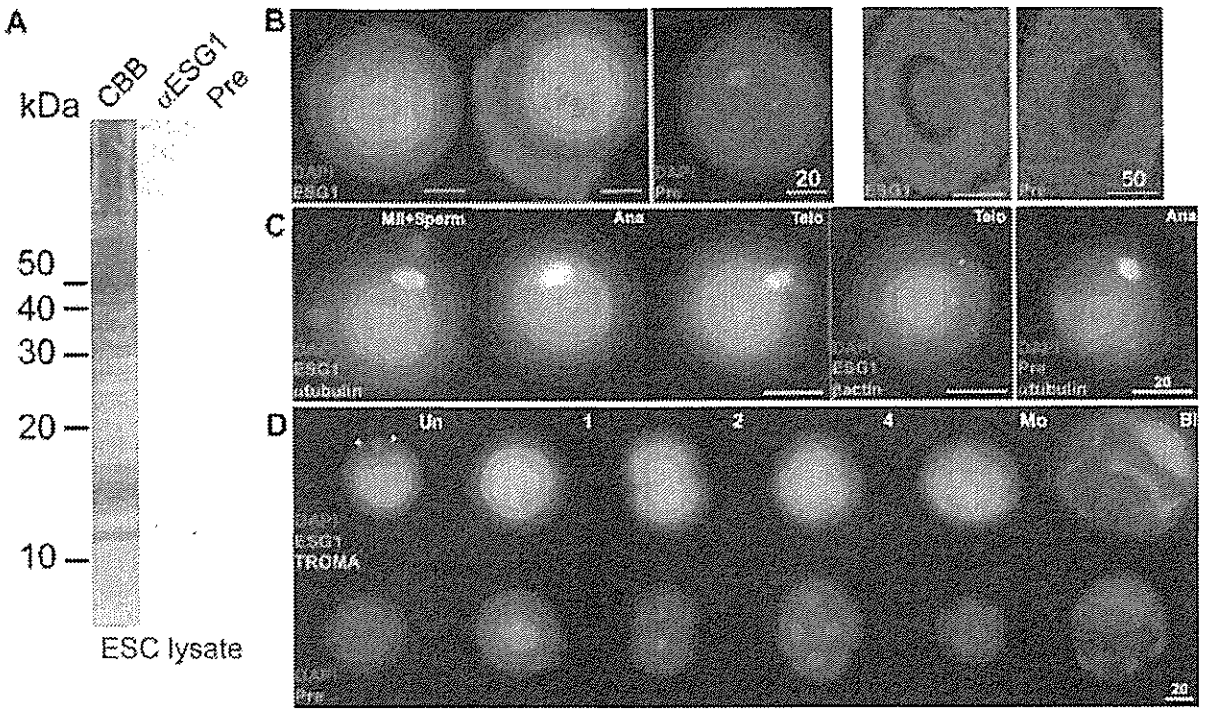

Fig. 2. Expression patterns and localization of ESG1 protein. (A) Crude lysate from embryonic stem (ES) cells was run on a 12.5% sodium dodecyl sulfate–polyacrylamide gel, followed by either CBB staining (CBB), or immunoblotting with αESG1 Pab (αESG1) or preimmune serum (Pre). A single band with a molecular 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

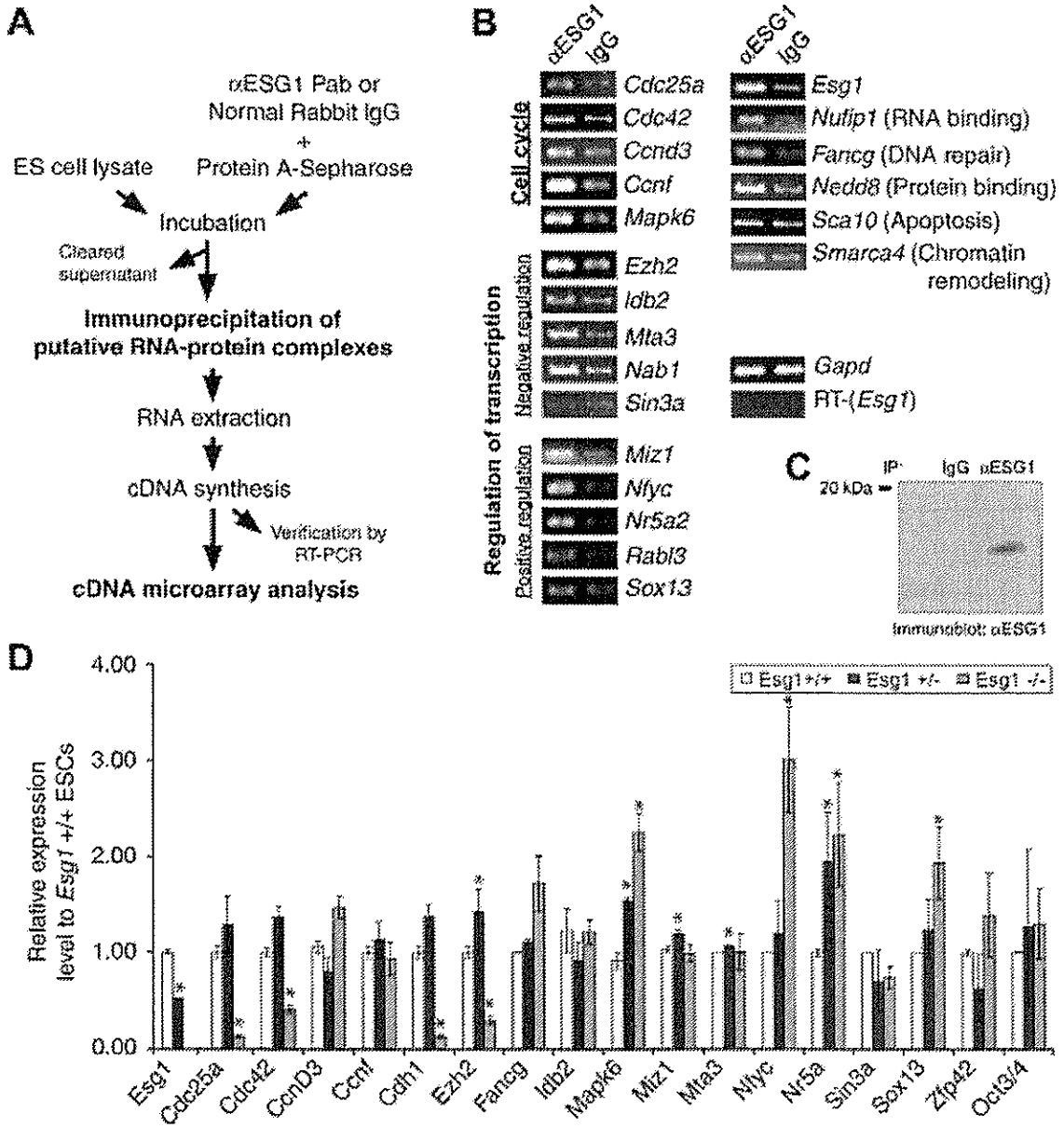

Fig. 3. Immunoprecipitation-microarray analysis identified binding targets of ESG1. (A) Schematic representation of immunoprecipitation (IP) followed by microarray analysis. See text for details. (B) Validation of IP-array results shown in (A). Relative to the control (IgG), enrichment of RNA that were 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 (*Ccnf*, *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.

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

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.

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	3468.60	<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	0.21036	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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[5] Human Embryonic Stem Cells

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Abstract

Human embryonic stem cells hold great promise in furthering our treatment of disease and increasing our understanding of early development. This chapter describes protocols for the derivation and maintenance of human embryonic stem cells. In addition, it summarizes briefly several alternative methods for the culture of human embryonic stem cells. Thus, this chapter provides a good starting point for researchers interested in harnessing the potential of human embryonic stem cells.

Introduction

In 1981, two groups succeeded in cultivating pluripotent cell lines from mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981). These cell lines, termed embryonic stem (ES) cells, originate from the inner cell mass (ICM) or epiblast and could be maintained *in vitro* without an apparent loss of developmental potential. The ability of these cells to contribute to all cell lineages has been demonstrated repeatedly both *in vitro* and *in vivo* (reviewed by Wobus and Boheler, 2005). Once established, ES cells display an almost unlimited proliferative capacity while retaining their developmental potential (reviewed by Smith, 2001). The first successful derivation of human ES (hES) cell lines was reported in 1998 (Thomson *et al.*, 1998). The establishment of hES cell lines provides a unique new research tool with widespread potential clinical applications.

Under specific *in vitro* culture conditions, hES cells also proliferate indefinitely without senescence and are able to differentiate into almost all tissue-specific cell lineages. These properties make hES cells an attractive candidate for cell replacement therapy and open exciting new opportunities to model human embryonic development *in vitro* (reviewed by Keller, 2005). In addition to developmental biology and cell-based therapy, the ES cell model has widespread applications in the areas of drug discovery and drug development (reviewed by Gorba and Allsopp, 2003).

Derivation of hES cell lines has not had a common uniform procedure among laboratories. Moreover, the culture and manipulation of hES cells differ considerably between laboratories and pose several unique challenges.

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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 1
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	30% 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	30% 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)