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Molecular Cloning and Functional Analysis of ESGP, an Embryonic Stem Cell and Germ Cell Specific Protein

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Abstract Several putative Oct-4 downstream genes from mouse embryonic stem (ES) cells have been identified using the suppression-subtractive hybridization method. In this study, one of the novel genes encoding an ES cell and germ cell specific protein (*ESGP*) was cloned by rapid amplification of cDNA ends. *ESGP* contains 801 bp encoding an 84 amino acid small protein and has no significant homology to any known genes. There is a signal peptide at the N-terminal of ESGP protein as predicted by SeqWeb (GCG) (SeqWeb version 2.0.2, http://gcg.biosino.org:8080/). The result of immunofluorescence assay suggested that *ESGP* might encode a secretory protein. The expression pattern of *ESGP* is consistent with the expression of *Oct-4* during embryonic development. ESGP protein was detected in fertilized oocyte, from 3.5 day postcoital (dpc) blastocyst to 17.5 dpc embryo, and was only detected in testis and ovary tissues in adult. *In vitro*, *ESGP* was only expressed in pluripotent cell lines, such as embryonic stem cells, embryonic carcinoma cells and embryonic germ cells, but not in their differentiated progenies. Despite its specific expression, forced expression of *ESGP* is not indispensable for the effect of *Oct-4* on ES cell self-renewal, and does not affect the differentiation to three germ layers.

Key words embryonic stem cell and germ cell specific protein (*ESGP*) gene; embryonic stem cell; Oct-4; self-renewal

Mouse embryonic stem (ES) cells are derived from the inner cell mass (ICM) of preimplantation blastocysts [1,2]. They can divide indefinitely in culture without differentiation and give rise to differentiated cell types that are derived from all three germ layers of the embryo (endoderm, mesoderm and ectoderm), both *in vivo* and *in vitro*. This ability, called pluripotency, is one of their defining features [3–5]. Several transcription factors, such as Oct-4 [6], Nanog [7,8] and STAT-3 [9,10] are essential for pluripotency maintenance.

The best characterized regulator of pluripotency is the transcription factor Oct-4, which belongs to class V of the Pit-Oct-Unc transcription factor family [6]. During mouse development, Oct-4 is expressed in pluripotent embryonic cells, such as blastomeres, ICM of blastocysts, epiblasts, primordial germ cells and most germ cells [11]. In line with its embryonic expression pattern, Oct-4 is found only in undifferentiated ES cells, embryonic carcinoma (EC) cells and embryonic germ (EG) cells *in vitro*, and is rapidly downregulated after their differentiation [12,13]. Inactivation of *Oct-4* in embryos and ES cells resulted in loss of pluripotency and differentiation into trophoblast lineage [6,14]. Conditional repression and induction in ES cells revealed that *Oct-4* can determine the differentiation, dedifferentiation and self-renewal state of ES cells in a sensitive and dose-dependent manner [15]. Thus there is a strong correlation between *Oct-4* gene expression and pluripotency maintenance.

To study how pluripotency is maintained, it is essential to know what genes are regulated by Oct-4. Oct-4 has been reported to regulate the expression of the *Rex-1* gene [16], the *Fgf-4* gene [17], the *Utf-1* gene [18], the *Fbx-15* gene [19], and so on. In our previous study, we reported

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the identification of putative downstream genes of Oct-4 in mouse ES cells by employing the suppression-subtractive hybridization method [20].

Here we describe the molecular cloning, expression and characterization of *ESGP*, a novel gene encoding an ES cell and germ cell specific protein.

Materials and Methods

Rapid amplification of cDNA ends (RACE)

The full-length cDNA of *ESGP* was cloned by both 5′- and 3′-RACE using the SMART-RACE cDNA amplification kit (BD Biosciences, Franklin Lakes, USA) according to the manufacturer's instructions. Briefly, first-strand cDNA for RACE reactions was reverse-transcribed from 1 μg poly(A) mRNA extracted from mouse ES-5 cells using the SMART II oligonucleotide and 5′- or 3′-RACE cDNA synthesis primer provided with the kit. The 5′- and 3′-RACE were performed by nested polymerase chain reaction (PCR) amplification. The primary 5′-RACE PCR reaction was performed with the universal primer mix (UPM) and gene-specific primer (5′-CCAGGACCCGTGGCCTCACTTCTG-3′). Subsequently, nested PCR reactions were performed using the nested universal primer (NUP) and gene-specific primer (5′-AACAGGCAGCAGCAGGCGGGACAGCAGC-3′). In a similar way, the 3′-RACE PCR reactions were first performed with UPM and gene-specific primer (5′-CCTGCCATGCCCGTTCCATTGCTC-3′) and then with NUP and gene-specific primer (5′-TGCTGTCCCGCCTGCTGCTGCCTGTTGC-3′). The full-length cDNA was then generated using the following primers: 5′-GATTCTGAGCAGTTCTGACTGGTG-3′ and 5′-CACTGTCCTCGTTTATTGACTACC-3′. All PCR products were gel-purified and sequenced.

Northern blot analysis

Total RNA was extracted from different types of cells using Trizol reagent (Invitrogen Life Technologies, San Diego, USA). From each sample, 30 μg RNA was separated on 1% agarose formaldehyde denaturing gel and transferred to a nylon membrane. Hybridization was performed at 42 °C overnight in hybridization solution containing formamide using the ^{32}P-labeled probes of the *ESGP* full-length cDNA fragment. Blots were washed twice with 0.2×standard saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) at room temperature for 30 min, twice with 0.2×SSC, 0.1% SDS at 42 °C for 30 min, then exposed to X-ray film at –80 °C with an intensifying screen for 72 h. The same blot was hybridized with ^{32}P-labeled mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA control probe.

Reverse transcription (RT)-PCR analysis

Total RNA was extracted from mouse embryos, two-month old adult tissue samples and cultured cells with Trizol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA was reverse-transcribed with oligo(dT) primer (Promega, Madison, USA) and StrataScript reverse transcriptase (Stratagene, San Diego, USA). PCR was carried out with ExTaq (TaKaRa, Tokyo, Japan). The following PCR primers were used: *ESGP* (5′-GATTCTGAGCAGTTCTGACTGGTG-3′) and (5′-CACTGTCCTCGTTTATTGACTACC-3′); *Oct-4* (5′-ACCTGGCTAAGCTTCCAAGG-3′) and (5′-GTGATCCTCTTCTGCTTCAG-3′); *Nanog* (5′-GGCTGATTTGGTTGGTGTC-3′) and (5′-ATCTGCTGGAGGCTGAGGTA-3′); *Gata-6* (5′-GCAATGCATGCGGTCTCTAC-3′) and (5′-CTCTTGGTAGCACCAGCTCA-3′); *Gata-4* (5′-GCCTGTATGTAATGCCTGCG-3′) and (5′-CCGAGCAGGAATTTGAAGAGG-3′); *Hnf1β* (5′-GAAAGCAACGGGAGATCCTCCGAC-3′) and (5′-CCTCCACTAAGGCCTCCCTCTCTTCC-3′); *LaminB1* (5′-CTGTTCTGAAAGTGAATGTGGTGGCCCC-3′) and (5′-GTTTAATCGCCTTCTCTGCTGCAACCTG-3′); *Dab2* (5′-GGCAACAGGCTGAACCATTAGT-3′) and (5′-TTGGTGTCGATTTCAGAGTTTAGAT-3′); *Afp* (5′-TCGTATTCCAACAGGAGG-3′) and (5′-AGGCTTTTGCTTCACCAG-3′); *Ttr* (5′-AGTCCTGGATGCTGTCCGAG-3′) and (5′-TTCCTGAGCTGCTAACACGG-3′); *Fgf-5* (5′-AAAGTCAATGGCTCCCACGAA-3′) and (5′-CTTCAGTCTGTACTTCACTGG-3′); *Brachyury T* (5′-ATGCCAAAGAAAGAAACGAC-3′) and (5′-AGAGGCTGTAGAACATGATT-3′); *GAPDH* (5′-ACCACAGTCCATGCCATCAC-3′) and (5′-TCCACCACCCTGTTGCTGTA-3′). The PCR products were fractionated by electrophoresis on 2% agarose gel.

Cell culture and differentiation

Mouse ES-5 and EG-4 cells were established in our laboratory [21], which were ES and EG cell lines, respectively, derived from 129 strain mice. P19 cells were an EC cell line kindly provided by Prof. C. L. MUMMERY (Institute for Development Biology, Utrecht, Netherlands). F9 cells were another EC cell line kindly provided by Prof. D. SOLTER (The Wistar Institute, Philadelphia, USA). All ES and EG cells were cultured at 37 °C with 5% CO_2 in Buffalo rat liver cell conditioned medium (BRL-CM) [21] or Dulbecco's modified Eagle's medium (DMEM)

supplemented with 15% fetal bovine serum (FBS), 0.1 mM β-mercaptoethanol and 1000 U/ml leukemia inhibitory factor (LIF) (Sigma, St. Louis, USA). P19 and F9 cells were cultured in DMEM supplemented with 10% FBS. For monolayer differentiation induced by retinoic acid (RA), ES-5, EG-4 and P19 EC cells were seeded at a density of 50,000 cells/ml and cultured in the presence of 0.1 μM RA for 6 d while F9 EC cells were induced with 1 μM RA. To grow embryoid bodies in suspension culture, ES-5 cells were seeded to bacterial culture plates containing BRL-CM and 1 nM RA at 1000 cells/cm^2. The medium was changed every other day during suspension culture. Embryoid bodies were harvested every 4 d up to day 12. For cell proliferation rate assay, 4000 cells were seeded in triplicate into gelatin-coated 96-well tissue culture dishes, 3-(4,5-dimenthyl-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed according to the standard method [22].

Plasmid construction and cell transfection

The expression plasmid pCBA-ESGP was constructed by inserting full-length *ESGP* cDNA into the hemagglutin (HA)-tagged vector pCBA-hrGFP, which was kindly provided by Dr. Sangmi CHUNG (Harvard Medical School, Massachusetts, USA) [23]. This vector drives high-level expression of the inserted gene, and the expression level is not influenced by cell induction with RA [21]. For cell transfection, ES-5 cells were transfected with the pCBA-ESGP construct using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and selected with ES medium containing 400 μg/ml G418 for 10 d. ES-5 cells transfected with the empty vector were used as a control.

Immunofluorescence assay

ES-5 cells transfected with *ESGP* cDNA expression plasmid were seeded onto slides pretreated with 0.1% gelatin, and fixed first in 4% paraformaldehyde for 15 min, then in methanol for 5 min at room temperature. The cells were washed three times in ice-cold phosphate-buffered saline (PBS) and permeabilised with 0.1% Triton/PBS for 10 min. The cells were then blocked with 3% bovine serum albumin (BSA)/PBS for 30 min at room temperature and incubated in primary anti-HA monoclonal antibody (Sigma) at 4 °C overnight. Goat anti-mouse IgG-fluorescein-isothiocyanate from Santa Cruz Biotechnology (Santa Cruz, USA) was used as secondary antibody.

Western blot analysis

Whole cell protein extracts from ES-ESGP cell colonies or control cells were fractioned by SDS-PAGE and immunoblotted onto nitrocellulose. Blots were incubated in primary anti-HA monoclonal antibody (1:10,000; Sigma) at room temperature for 1 h and washed with 0.1% Tween-20/PBS. Goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used at 1:2000 dilution and blots were developed using a SuperSignal west pico kit (Pierce, Rockford, USA).

Self-renewal efficiency assay

To measure self-renewal of ES cells, ES-5V or ES-ESGP cells were plated at 1000 cells per well in six-well cell culture dishes and maintained in ES medium containing RA or LIF in different concentrations. On day 6, the colonies were washed, fixed and stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) for alkaline phosphatase activity. Numbers of undifferentiated and differentiated colonies were counted under the microscope. The numbers of undifferentiated colonies of ES-5V cells in 1000 U/ml LIF were defined as 100% during data analysis. Alkaline phosphatase positive colonies were counted in triplicate for each treatment.

Teratoma analysis

ES-ESGP cells and ES-5V cells were harvested at 10^7 cells/ml and 0.5 ml was injected subcutaneously into the right flank of Balb/Balc nude mice. After 1 month, teratomas were harvested, fixed in Bouin's solution and embedded in wax. Sections were made and stained using the hematoxylin-eosin method.

Results

Cloning and sequence analysis of mouse *ESGP* cDNA

Based on the partial sequence of *ESGP* obtained from our previous study [20], both 5′- and 3′-RACE were carried out as described in "Materials and Methods". The resulting *ESGP* cDNA (GenBank accession No. DQ190000) was 801 bp in length, which corresponds well with the 0.8 kb size estimated from Northern blots. The sequence contained an open reading frame (ORF) of 255 bp according to the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) (**Fig. 1**). The ATG at position 104 of the transcript was assigned as the initiation codon and the poly(A) signal sequence AATAAA was located 420 bp downstream of the termination codon. *ESGP* cDNA sequence was searched against the GenBank databases by BLAST (http://www.

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                                                GAAATTGATTCTG
  14  AGCAGTTCTGACTGGTGAGAGCTGCCACTGGCCGGTTAGAACTGG
  59  TGAGCAGGAGGGCAAGAAGTTCAGGCTTCAGGTGCAGGTCCTGCC
 104  ATGCCCGTTCCATTGCTCCCGATGGTGCTTCGATCGCTGCTGTCC
   1   M  P  V  P  L  L  P  M  V  L  R  S  L  L  S
 149  CGCCTGCTGCTGCCTGTTGCCCGCCTGGCCCGGCAGCACCTCCTG
  16   R  L  L  L  P  V  A  R  L  A▼R  Q  H  L  L
 194  CCCTTGCTGCGCCGGCTGGCCCGCCGACTGAGCTCCCAAGACATG
  31   P  L  L  R  R  L  A  R  R  L  S  S  Q  D  M
 239  AGAGAGGCTCTGCTGAGCTGTCTGCTCTTTGTCCTCAGCCAGCAA
  46   R  E  A  L  L  S  C  L  L  F  V  L  S  Q  Q▼
 284  CAGCCACCGGATTCTGGAGAGGCCTCCAGAGTGGACCACTCCCAG
  61   Q  P  P  D  S  G  E  A  S  R  V  D  H  S  Q
 329  AGGAAGGAGAGATTGGGCCCCCAGAAGTGAGGCCACGGGTCCTGG
  76   R  K  E  R  L  G  P  Q  K  *
 374  AAACAGCAACGCCCATCAAAGTACTTTTGGAGCCGGTTAGTCCAG
 419  GCGTCGGTCCGCACGCACGGGCATGGACGGCAGACTGCCCAGTGG
 464  CGAAGACAGTCCGGGCTGAGTGCAAGAGGGCTCTGACCTGAACAG
 509  TTCCCATTCCCCTAGCTCCTAGCAGGCTACAGATTGTGTTGATTG
 554  ACCCTTCCTCTTTGCAGCTCCCTCTGCCTTATTTCTGGCCTCCAG
 599  GGTGGACCTGCAAATGCGGGTATCAAGGTCAGTTAAGAGATGATG
 644  TTTCACTTAGACTCAAGACAATTTAGCTAAGAGGTGGTATTTAAA
 689  TCCAAACTTTCCCGTCTACCTTAAAATTATAAGCCCTTGCTTCCC
 734  GTTAAAGAATGAGTACTTTCAAAGCTTGGTACGCAGCAGGTAGTC
 779  AATAAACGAGGACAGTGTGTTGT
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Fig. 1 Nucleotide and deduced amino acid sequence of the gene encoding murine embryonic stem cell and germ cell specific protein

▼, potential signal peptide cleavage sites at amino acid positions 25 and 60. The polyadenylation signal is double underlined.

ncbi.nlm.nih.gov/BLAST), which showed no significant homology to any known genes, but showed high similarity with one hypothetical gene (GenBank accession No. XM_488821), which is 99% identical to *ESGP* at the nucleotide sequence level and on mouse chromosome 17.

ESGP may encode a secretory protein

ESGP encodes an 84 amino acid protein. Protein sequence analysis using SeqWeb (GCG) (SeqWeb version 2.0.2, http://gcg.biosino.org:8080/) showed no significant homology to any conserved protein domain, but only a possible signal peptide. There are two potential signal peptide cleavage sites at amino acid positions 25 and 60, which suggest that *ESGP* may encode a small secretory protein. Because we were not successful in generating the antibody with a synthetic C-terminal peptide (data not shown), we examined the intracellular localization of ESGP protein by fusing the HA-tag at the C-terminal of ESGP. The ESGP cells were established by transfecting HA-tagged ESGP expression plasmid and were immunostained with anti-HA monoclonal antibody. The control ES-5V cells, transfected with an empty vector, did not show any staining of HA antigen. However, ESGP cells showed the localization of ESGP protein was mainly in the cytoplasm and near the cell membrane, but not in the nucleus (**Fig. 2**). Western blot showed a strong hybridized band, but because it was so small it was difficult to accurately determine its size (**Fig. 3**). These data suggest *ESGP* may encode a secretory protein.

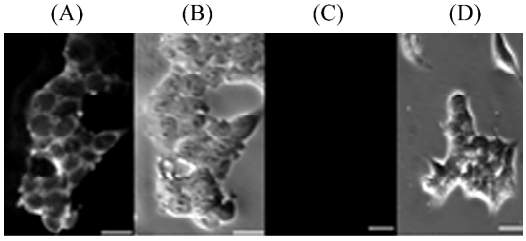

Fig. 2 Immunofluorescence localization of an HA-tagged embryonic stem cell and germ cell specific protein (ESGP) in ES-5 cells

The ESGP protein was mainly in the cytoplasm and near the cell membrane, but not in the nucleus. Bright field images (B,D) and fluorescence images of the same samples (A,C) are shown. (A,B) ESGP (ES-5 cells transfected with the pCBA-ESGP construct) cells. (C,D) ES-5V (ES-5 cells transfected with the empty vector) cells. Bar=50 μm.

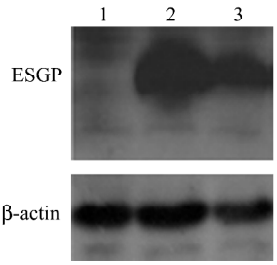

Fig. 3 Western blot analysis of embryonic stem cell and germ cell specific protein (ESGP) expression with anti-HA antibody

1, ES-5V (ES-5 cells transfected with the empty vector) cells; 2, undifferentiated ES-ESGP (ES-5 cells transfected with the pCBA-ESGP construct) cells; 3, differentiated ES-ESGP cells. β-actin was used as the control.

Expression pattern of *ESGP in vivo* and *in vitro*

The expression pattern of *ESGP* during embryo development was examined by RT-PCR analysis using mouse embryos at different stages. The expression of *ESGP* was weakly detected in fertilized oocyte, upregulated in 3.5 day postcoital (dpc) blastocyst and sustained in later-stage embryo until 17.5 dpc (data not shown) [**Fig. 4(A)**]. In several adult mouse tissues examined, *ESGP* could only be detected in testis and ovary [**Fig. 4(B)**]. Notably, the

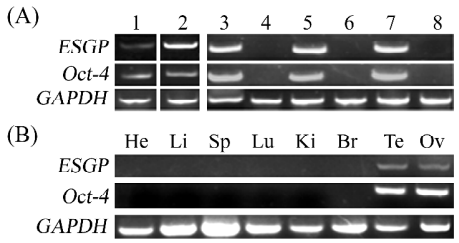

Fig. 4 Reverse transcription-polymerase chain reaction analysis of embryonic stem cell and germ cell specific protein (*ESGP*) gene expression in embryos, cell lines and adult tissues at different developmental stages

(A) Expression of *ESGP* in different developmental stage embryos and cell lines. 1, fertilized oocytes; 2, blastocysts; 3, undifferentiated ES-5 cells; 4, differentiated ES-5 cells; 5, undifferentiated EG-4 cells; 6, differentiated EG-4 cells; 7, undifferentiated F9 cells; 8, differentiated F9 cells. (B) Expression of *ESGP* in adult tissues. He, heart; Li, liver; Sp, spleen; Lu, lung; Ki, kidney; Br, brain; Te, testis; Ov, ovary. *GAPDH* was used as the control.

expression pattern of *ESGP* is completely consistent with the expression of *Oct-4* during development, suggesting *ESGP* is the downstream gene of *Oct-4*.

We then examined the expression of *ESGP* in several *in vitro* cell lines by both RT-PCR [**Fig. 4(A)**] and Northern blot (**Fig. 5**). *ESGP* was expressed in undifferentiated ES cells, EG cells and F9 EC cells. Upon differentiation induced with RA, *ESGP* expression was lost. We could not detect the expression of *ESGP* in P19 EC cells. These results demonstrate that the expression of *ESGP* appears restricted to pluripotent cell lines as same as *Oct-4*.

ESGP is dispensable for ES cell self-renewal

ESGP is the downstream gene regulated by Oct-4 in ES cells. To examine whether *ESGP* transduces the effect of *Oct-4* on ES cell self-renewal, we established the ES cell line with sustained high expression of *ESGP*. This expression level was not influenced by cell induction with RA [**Fig. 6 (A)**]. When cultured in ES cell medium, ES-ESGP cells had the same colony morphology as control ES-5V cells [**Fig. 6(B)**]. Both cells retained positive alkaline phosphatase activity (data not shown), and MTT analysis showed the similar proliferation rate [**Fig. 6(C)**]. These results suggest that the sustained expression of *ESGP* does

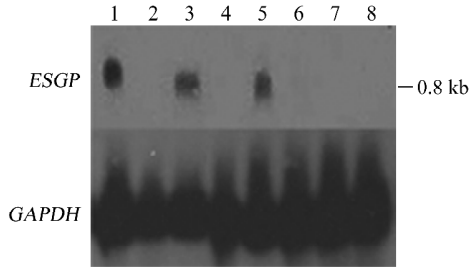

Fig. 5 Northern blot analysis of embryonic stem cell and germ cell specific protein (*ESGP*) gene expression in different cell lines

1, undifferentiated ES-5 cells; 2, differentiated ES-5 cells; 3, undifferentiated EG-4 cells; 4, differentiated EG-4 cells; 5, undifferentiated F9 cells; 6, differentiated F9 cells; 7, undifferentiated P19 cells; 8, differentiated P19 cells.

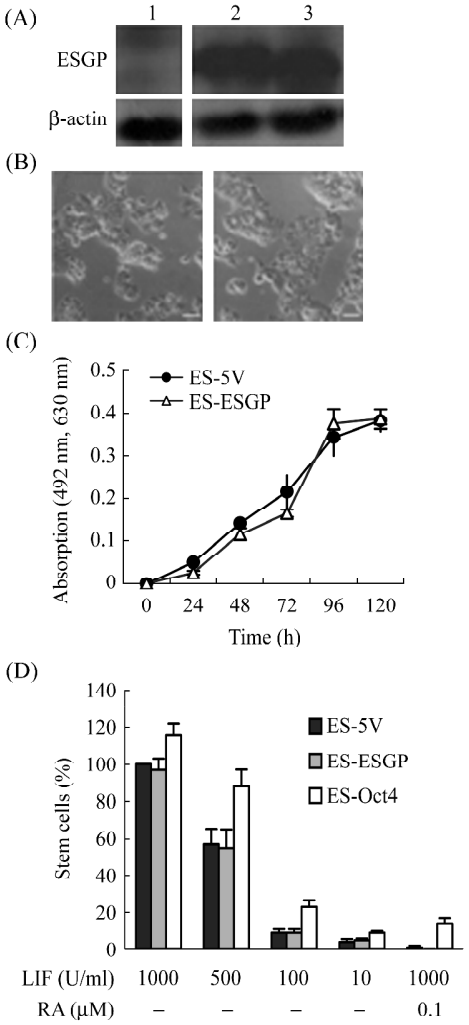

Fig. 6 Comparison of morphology, proliferation rate and efficiency of self-renewal of ES-ESGP cells and control ES-5V cells

(A) Western blot analysis of the expression of ESGP transgene product in ES-5V (ES-5 cells transfected with the empty vector) cells and ES-ESGP (ES-5 cells transfected with the pCBA-ESGP construct) cells. 1, ES-5V cells; 2, undifferentiated ES-ESGP cells; 3, differentiated ES-ESGP cells. (B) Phase-contrast image of control ES-5V cells (left) and ES-ESGP cells (right). Bar=50 μm. (C) Proliferation rate assay of control ES-5V cells and ES-ESGP cells. (D) Efficiency of self-renewal of ES-ESGP cells, ES-Oct4 cells and control ES-5V cells.

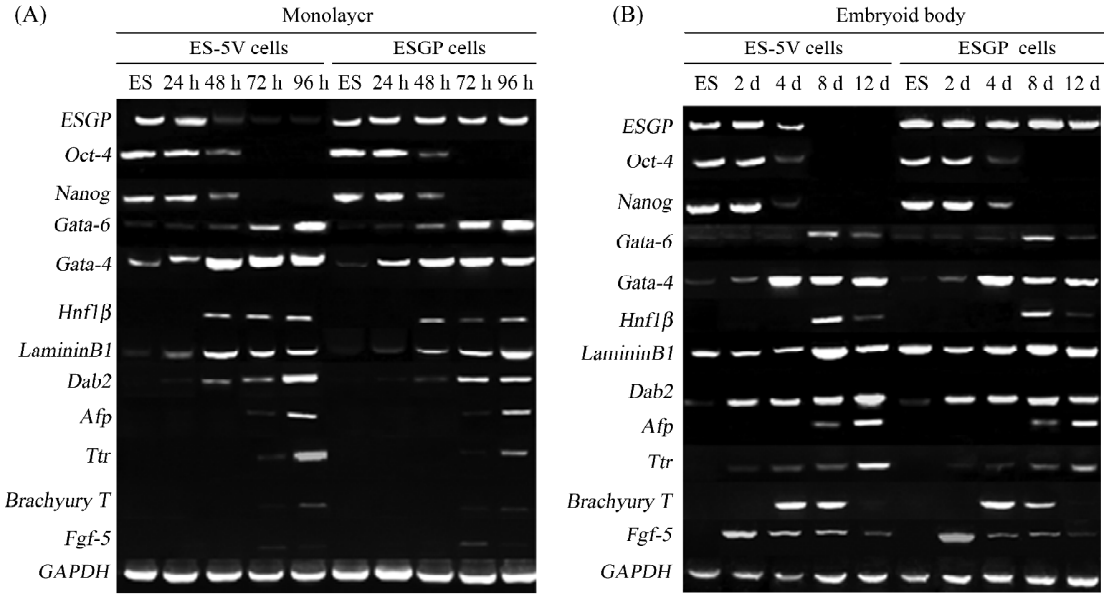

Fig. 7 Lineage-specific gene expression during ES-5V and ES-ESGP cell differentiation in monolayer (A) or embryoid body (B) condition

Expression of the marker gene was determined by reverse transcription-polymerase chain reaction. Embryonic stem (ES) cells were induced to differentiation with retinoic acid for 24, 48, 72, and 96 h in a monolayer or grown as embryoid body for up to 12 d. ES-5V cells, ES-5 cells transfected with the empty vector; ES-ESGP cells, ES-5 cells transfected with the pCBA-ESGP construct.

not change the character of ES-5 cells. The efficiency of self-renewal of ES cells was then assayed. When the concentration of LIF was reduced, the numbers of undifferentiated colonies of ES-ESGP and control ES-5V cells were both reduced to the same extent. When induced by 0.1 μM RA, neither the ES-ESGP cells nor the control ES-5V cells could form stem cell colonies, indicating that, unlike *Oct-4*, *ESGP* alone is not able to maintain the undifferentiation state of ES cells [**Fig. 6(D)**].

ESGP has no obvious effect on ES cell differentiation *in vitro* or *in vivo*

As described above, the forced expression of *ESGP* does not change the morphology, proliferation or LIF-dependent character of ES cells. We then examined whether *ESGP* influences ES cell differentiation. When induced with RA, they differentiated normally under the conditions of a monolayer or embryoid bodies. There were no obvious differences on the expression of marker genes of the three germ layers, such as ectoderm marker *Fgf-5* gene, mesoderm marker *Brachyury T* gene, or endoderm marker *Gata-6*, *Gata-4*, *Afp* and *Ttr* genes (**Fig. 7**). When injected subcutaneously into nude mice, they formed teratomas consisting of all the cell types of three germ layers (**Fig. 8**). These results indicate that *ESGP* has no obvious effect on

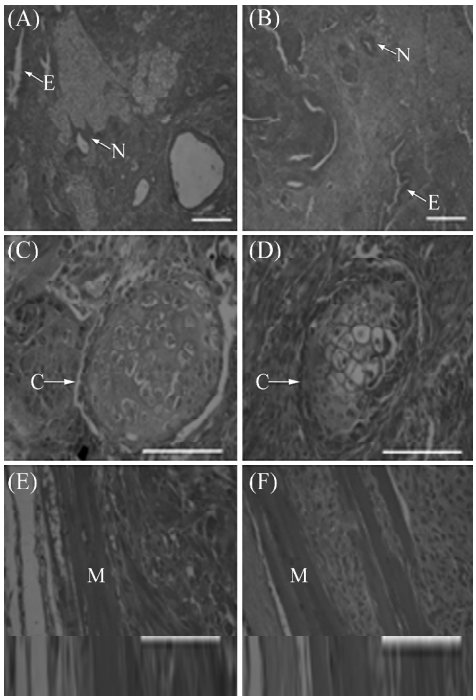

Fig. 8 Histological analysis of teratomas derived from ES-5V cells (A,C and E) and from ES-ESGP (B,D and F) cells

E, epithelia; C, cartilage; M, muscle; N, neuron tube. ES-5V cells, ES-5 cells transfected with the empty vector; ES-ESGP cells, ES-5 cells transfected with the pCBA-ESGP construct. Bar=50 μm.

ES cell differentiation *in vitro* or *in vivo*.

Discussion

Transcription factor Oct-4 plays a critical role in maintaining the pluripotency and self-renewal of ES cells. However, only a few Oct-4 downstream genes have been identified. In this study, we characterize a novel Oct-4 downstream gene, *ESGP*. The *ESGP* cDNA is 801 bp in length and encodes an 85 amino acid small protein. Nucleotide and protein sequence analysis showed no significant homology to any known genes. It is notable that the expression pattern of *ESGP* is consistent with the expression of *Oct-4* in early embryonic blastocyst and is confined to the *in vitro* pluripotent cell lines, such as ES, EG and EC cell lines, but not their differentiated progenies. However, despite its specific expression pattern, *ESGP* is dispensable for ES cell self-renewal and differentiation. The ES cells with forced expression of *ESGP* were normal in self-renewal and differentiated normally into the cells of all three germ layers both *in vitro* and *in vivo*. These data suggest that *ESGP* does not transduce the effect of *Oct-4* to maintain the pluripotent status of ES cells. Several Oct-4 downstream genes were recently identified in ES cells, such as *Fbx15* [19] and *Zfp-57* [24]. However, none is related to the function of Oct-4 in ES cell renewal, suggesting that the real target gene of Oct-4 in ES cells has not been identified, or that Oct-4 may regulate a group of genes to exert its function cooperatively and redundantly.

Oct-4 not only plays a critical role in maintaining the pluripotency of ES cells, but also has an essential role in maintaining the viability of the mammalian germline. Loss of Oct-4 function leads to apoptosis of primordial germ cells [25]. *ESGP* is expressed in embryonic germ cells and in reproductive tissues testis and ovary accompanying *Oct-4*, implying that *ESGP* may transduce the effect of *Oct-4* in germ cells. Interestingly, *ESGP* encodes a small secretory protein. There are many small secretory proteins involved in germ cell development, such as gonadotrophin, luteinizing hormone and follicle-stimulating hormone in stimulating follicular development [26], testatin in testis development [27]. Further analysis, including the generation of *ESGP*-deficient mice and locating *ESGP* expression in detail in the reproductive system, will contribute to the understanding of the function of the *ESGP* gene in germ cell development.

In summary, we described here the molecular cloning and functional analysis of an embryonic stem cell and germ cell specific protein, ESGP. Its specific expression pattern is consistent with the expression of *Oct-4*, suggesting *ESGP* is the downstream gene of *Oct-4*. However, forced expression of *ESGP* has no obvious effect on ES cell self-renewal or differentiation, and does not transduce the effect of *Oct-4* to maintain the pluripotent status of ES cells.

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References

1 Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981, 292: 154–156
2 Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma. Proc Natl Acad Sci USA 1981, 78: 7634–7638
3 Bradley A, Evans M, Kaufman MH, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 1984, 309: 255–256
4 Smith AG. Embryo-derived stem cells: Of mice and men. Annu Rev Cell Dev Biol 2001, 17: 435–462
5 Smith AG. Embryonic stem cells. In: Marshak DR, Gardner RL, Gottleib D eds. Stem Cell Biology. New York: Cold Spring Harbor Laboratory Press 2001
6 Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H *et al*. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 1998, 95: 379–391
7 Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M *et al*. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 2003, 113: 631–642
8 Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003, 113: 643–655
9 Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev 1998, 12: 2048–2060
10 Matsuda T, Nakamura T, Nakao K, Arai T, Katsuki M, Heike T, Yokota T. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. EMBO J 1999, 18: 4261–4269
11 Pesce M, Gross MK, Scholer HR. In line with our ancestors: Oct-4 and the mammalian germ. Bioessays 1998, 20: 722–732
12 Okamoto K, Okazawa H, Okuda A, Sakai M, Muramatsu M, Hamada H. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell 1990, 60: 461–472
13 Yeom YI, Fuhrmann G, Ovitt CE, Brehm A, Ohbo K, Gross M, Hubner K *et al*. Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 1996, 122: 881–894
14 Hay DC, Sutherland L, Clark J, Burdon T. Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and

mouse embryonic stem cells. Stem Cells 2004, 22: 225–235

15 Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 2000, 24: 372–376

16 Ben-Shushan E, Thompson JR, Gudas LJ, Bergman Y. Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. Mol Cell Biol 1998, 18: 1866–1878

17 Schoorlemmer J, Kruijer W. Octamer-dependent regulation of the kFGF gene in embryonal carcinoma and embryonic stem cells. Mech Dev 1991, 36: 75–86

18 Nishimoto M, Fukushima A, Okuda A, Muramatsu M. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol Cell Biol 1999, 19: 5453–5465

19 Tokuzawa Y, Kaiho E, Maruyama M, Takahashi K, Mitsui K, Maeda M, Niwa H *et al*. Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. Mol Cell Biol 2003, 23: 2699–2708

20 Du Z, Cong H, Yao Z. Identification of putative downstream genes of Oct-4 by suppression subtractive hybridization. Biochem Biophys Res Commun 2001, 282: 701–706

21 Du ZW, Cong XQ, Yao Z. Forced expression of the Oct-4 gene influences the differentiation of embryonic stem cells. Chinese Science Bulletin 2001, 46: 1446–1449

22 Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983, 65: 55–63

23 Chung S, Andersson T, Sonntag KC, Bjorklund L, Isacson O, Kim KS. Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines. Stem Cells 2002, 20: 139–145

24 Akagi T, Usuda M, Matsuda T, Ko MS, Niwa H, Asano M, Koide H *et al*. Identification of Zfp-57 as a downstream molecule of STAT3 and Oct-3/4 in embryonic stem cells. Biochem Biophys Res Commun 2005, 331: 23–30

25 Kehler J, Tolkunova E, Koschorz B, Pesce M, Gentile L, Boiani M, Lomeli H *et al*. Oct4 is required for primordial germ cell survival. EMBO Rep 2004, 5: 1078–1083

26 Richards JS. Hormonal control of gene expression in the ovary. Endocr Rev 1994, 15: 725–751

27 Tohonen V, Osterlund C, Nordqvist K. Testatin: A cystatin-related gene expressed during early testis development. Proc Natl Acad Sci USA 1998, 95: 14208–14213

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