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Identification, expression and subcellular localization of *ESRG*

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A B S T R A C T

ESRG (embryonic stem cell related gene, also known as *HESRG*), is a novel human gene first cloned and identified by our group with microarray analysis. Interestingly, it is expressed specifically in undifferentiated human embryonic stem cells (hESCs), while its expression pattern and its role in hESCs remain unclear. Here, full-length 3151nt *ESRG* cDNA was further identified by RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) technique. Meanwhile, an alternatively splicing *ESRG* transcript (*ESRG-B*) of 2837nt in length was also found. Surprisingly, bioinformatics analyses showed that the open reading frames (ORFs) of *ESRG* and *ESRG-B* were identical. Both of them consist of 669nt and encode a 222aa protein with a predicted molecular size of 24 kDa. The ESRG protein was located in the nuclei of hESCs as demonstrated by immunocytochemical staining and Western blotting using ESRG specific antibody generated by us. In contrast, ESRG located in the cytoplasm of COS7 cells when it was forced to be expressed in these cells by gene transfection strategy, suggesting there may be some special proteins present only in hESCs which can help ESRG protein transport into the nuclei of hESCs. By spatial expression analysis, we further discovered that *ESRG* only expressed in the ovary tissue and hESCs instead of other tissues or cell lines. Our current data provide us with an important basis for conducting further studies on the functions and regulatory mechanisms underlying the role of *ESRG* in hESCs.

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1. Introduction

Embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass of a pre-implantation blastocyst, an early-stage embryo. ESCs can be induced to differentiate into different cell lineages *in vitro* [1]. Thus, it provides us an experimentally tractable *in vitro* system to understand the molecular programs that control the earliest steps of lineage specification and maintenance of pluripotency. Oct4, Sox2, and Nanog are often described as the core set of transcription factors necessary for maintaining the pluripotency of ESCs [2]. However, not all the genes related to the pluripotency of ESCs have been discovered [3,4].

ESRG, a novel human embryonic stem cell related gene, is located at chromosome 3p14.3 and composed of four exons and three introns with a full-length mRNA of 3151 nucleotides (nt). It only expressed in undifferentiated hESCs but not in their differentiated ones [5]. Presumably, it may play an important role in the maintenance of self-renewal or pluripotency of hESCs, possibly through involvement in the core regulatory network consisting of *Oct4*, *Sox2*, *Nanog*, *etc*. Bioinformatics analysis shows that *ESRG* gene may contain an open reading frame (ORF) comprising 669nt with a CTG initiation codon at 2–4nt and a stop codon at 668–670nt, and encode a 24 kDa protein containing 222 amino acid (aa) residues. It is predicted to be located in the nuclei. In this report, we used expression strategy to identify the ORF and subcellular localization of ESRG. Meanwhile, we detected *ESRG* expression in various tissues to profile its spatial expression pattern. As far as we know, this is the first report to show the ORF and subcellular localization of ESRG, and our current data provides us with an important basis for conducting further studies on the special role of *ESRG* in hESCs and the underlying molecular mechanisms.

1.1. Cell culture

Human ES cells (H9 and H1 cell lines) were from WiCell Research Institute (Madison, WI, USA) and cell culture was performed as described previously [6,7]. Briefly, hESCs were cultured on mitomycin-C treated mouse embryonic fibroblast (MEF) feeder layer in DMEM/F12 medium (Invitrogen, USA) supplemented with 20% knockout serum replacement (KSR) (a serum-free formulation)

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(Invitrogen), 1 mM glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma, USA), 1% nonessential amino acids (Invitrogen), and 8 ng/ml basic fibroblast growth factor (bFGF, PeproTech, USA). To obtain a feeder-free culture, the cells were cultured on Matrigel (BD Bioscience, USA) and grown in media conditioned for at least 24 h by MEFs. C57BL/6 mouse ES cell line (ATCC, USA) was maintained in DMEM (Invitrogen) supplemented with 15% FBS (Gibco), 0.1 mM β-mercaptoethanol, 1 mM glutamine, 1% nonessential amino acids and 1,000 units/ml of leukemia inhibitory factor (LIF) (Millipore, USA). All regular media were supplemented with 10% FBS for other cell lines. SK-OV-3 (human ovarian carcinoma cell line) was cultured in DMEM/F12 medium (Invitrogen). HeLa (human cervical carcinoma epithelial cell line) was grown in minimum essential medium (MEM) (Hyclone, USA). HEK-293 (human embryonic kidney cell line), COS7 (African green monkey kidney cell line) and NIH/3T3 (mouse embryonic fibroblast cell line) were grown in DMEM. JAR (human choriocarcinoma cell line) was grown in RPMI 1640 (Invitrogen). These cell lines were kept in our lab.

1.2. Tissue samples

Seven kinds of human fetal tissues (including skin, lung, brain, thyroid, ovary, thymus, liver) of aborted fetus (36 weeks old) and adult testis tissue were obtained with informed content and the study was performed with approval of the ethics committee of each institution involved in this project.

1.3. 5′- and 3′-rapid amplification of cDNA ends (RACE)

Prior to this report, the 5′-end of ESRG was obtained using SMART RACE cDNA Amplification Kit (Clontech). However, the main limitation of this classic RACE technique is that there is no selection for amplification of fragments corresponding to the actual 5′-ends of mRNA: all cDNAs are acceptable templates in polymerase chain reaction (PCR). RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) is designed to amplify cDNA only from full-length, capped mRNA, usually producing a single band after PCR, so its result is more reliable [8].

Thus, we amplified the 5′-end of ESRG using a commercially available RLM-RACE kit (GeneRacer™ Kit, Invitrogen). Briefly, 2–5 μg DNase I-treated RNA was dephosphorylated with calf intestine phosphatase, digested by tobacco acid pyrophosphate (TAP) to remove the 5′cap structure, and ligated to 5′ RACE adapter at 5′-end using T4 RNA ligase. The ligated RNA was transcribed into cDNA with random primer and used as a template for the PCR reaction with GeneRacer 5′primer and 5′-GSP (Table 1). Each PCR was run in 50 μl of reaction volume containing 5 μl of 10 × PrimeSTAR™ buffer (Mg^{2+} plus), 4 μl of dNTPs mixture (2.5 mM each), 1 μl of each primers (10 mM), 2 μl of 5′-RACE-Ready cDNA, 0.5 μl PrimeSTAR™ HS DNA polymerase (2.5 U/μl) and 36.5 μl PCR-grade water. The touchdown PCR conditions were 94 °C for 3 min, 5 cycles (94 °C for 30 s, 72 °C for 1 min), 5 cycles (94 °C for 30 s, 70 °C for 1 min), 22 cycles (94 °C for 30 s, 68 °C for 30 s, 72 °C for 1 min), and then a final extension at 72 °C for 10 min.

The 3′-end of the cDNA was amplified by SMART RACE cDNA Amplification Kit (Clontech). Briefly, 2–5 μg DNase I-treated RNA was transcribed into cDNA with 3′ RACE adapter and used as a template for the PCR reaction. The touchdown PCR was performed with UPM (Universal Primer Mix) and 3′-GSP (Table 1) under the same condition as 5′ RACE PCR.

Both PCR products for 5′-end and 3′-end were electrophoresed in a 1.5% agarose gel and purified from the gel using Qiaquick Gel Extraction Kit (Qiagen, Germany). The purified PCR products were subcloned into the pMD18-T vector (TaKaRa, Japan). Plasmid DNA was extracted with Plasmid Mini Purification Kit (Qiagen) and sequenced by Invitrogen Biotech Co. Ltd. (Shanghai, China). Two primers complementary to the T7 and SP6 promoters of the vector were used for sequencing. Ten independent clones of each PCR product were analyzed to avoid errors in sequence analysis.

The complete sequence of the *ESRG* cDNA was deduced from the overlapping sequences of both 5′-end and 3′-end amplification products. All oligonucleotides used in this study were synthesized by Invitrogen Biotech Co. Ltd..

Table 1
Primers used in this study.

Primer name	Primer sequence (5′ to 3′)
RACE-PCR primers	
5′-GSP	TAGTGAGGGAGGTTGGAGAACAGAGTA
GeneRacer 5′primer	CGACTGGAGCACGAGGACACTGA
3′-GSP	AAGCAGTGGTATCAACGCAGAGT
UPM	ctaatacgactcactatagggcAAGCAGTGGTATCAACGCAGAGT
ORF cloning primers	
ORF sense	CTGACTCTCTTTTCGGACTCAG
ORF antisense	TGAAAATAAGCGATTGGGGGGTT
Gene-specific primers for RT-PCR	
GAPDH forward	CTTTGGTATCGTGGAAGGACTC
GAPDH reverse	CTCTTCCTCTTGTGCTCTTGCT
ESRG forward	ATGAAAGGGAAGACATACAA
ESRG reverse	TGAACATAGCAAGGGAAA
ESRG-B forward	TGAAAGGGAAGACATACAAAAAC
ESRG-B reverse	GAACATCTCCAGAACAACCTCACAG

1.4. Sequence analysis

The similarity analyses of the determined nucleotide sequences and deduced amino acid sequences were performed by BLAST programs <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The prediction of transcription start sites was conducted using TSS finder <http://www.fruitfly.org/seq_tools/promoter.html> [9]. The possible ORF was analyzed with ORF Finder tool <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>. The protein sequence of the cloned gene, its molecular weight, pI and topology predictions were conducted with the Expert Protein Analysis System <http://expasy.org/tools/> [10]. The simple modular architecture research tool (SMART) version 4.0 <http://smart.embl-hidelberg.de/> was used to predict the protein domains [11]. The subcellular localization of ESRG protein was predicted using ngLOC software <http://ngloc.unmc.edu/index.html> [12].

1.5. Reverse transcription-PCR (RT-PCR)

All PCR primers used in this study were summarized in Table 1. Total RNA was extracted with Trizol reagent (Invitrogen) and digested by the RNase-free DNase I (TaKaRa) to remove trace amounts of genomic DNA contamination. The concentration and quality of total RNA were determined by UV-absorbance at 260 nm, the A260/A280 ratio and agarose gel electrophoresis. The first-strand cDNA was synthesized from total RNA using reverse transcription system (Promega, USA) and subjected to PCR (95 °C for 5 min; 30 cycles through 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and then extension at 72 °C for 10 min) using DreamTaq Green PCR Master Mix (Thermo, USA) with primers listed in Table 1. *GAPDH* was used as an internal loading control. Controls without template were carried out by replacing cDNA with water. PCR products were analyzed in 1.5% agarose gels and visualized by ethidium bromide staining.

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