Biochemical and Biophysical Research Communications 460 (2015) 130–135

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

An ESRG-interacting protein, COXII, is involved in pro-apoptosis of human embryonic stem cells

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ARTICLE INFO

Article history:
Received 14 February 2015
Available online 3 March 2015

Keywords:
hESC
ESRG
COXII
Mitochondrial apoptosis

ABSTRACT

Human embryonic stem cells(hESC) posses very promising application perspective in clinical transplant therapies for their characteristics of self-renewal and pluripotency. So efforts focusing on the mechanisms of the two characteristics are extremely important. *ESRG*, first identified by our group, is a candidate stemness gene of hESC for its much higher expression level in hESC comparing to that in 7-day embryoid bodies(EBs). Here, the proteins interacted with ESRG and its functions in hESC were explored. Yeast two-hybrid (Y2H) screening system was adopted to explore the interacting proteins of ESRG. Then Co-IP was performed to confirm the interactions between candidate proteins and ESRG. At last, the functions of validated interacting protein were explored by RNA interference(RNAi) and Western blot(WB). There were no autonomous activation and toxicity in the Y2H system, which verified its availability. Four candidate proteins, AAMP, DDT, GNB2L1 and COXII, were discovered, and the interaction between ESRG and COXII was ultimately confirmed. The expression of COXII in hESC was suppressed by siRNA, and the inhibited mitochondrial apoptosis was observed in hESC with downregulated COXII expression. Our work first validated the interaction between ESRG and COXII, and demonstrated that COXII serves as a pro-apoptotic protein in hESC. The results implied that ESRG may play an important role in regulating the apoptosis of hESC by interacting with COXII, and thus contribute a lot to the maintenance of hESC characteristics.

1. Introduction

Human embryonic stem cells (hESCs), usually isolated from inner cell mass of blastocyst, have tremendous potential in clinical applications like cell replacement therapy, tissue or even organ transplantation for their two hallmarks: self-renewal and pluripotency [1–3]. Self-renewal means hESCs can maintain their population and characteristics by unlimited symmetric cell divisions under proper conditions both *in vivo* and *in vitro*. Pluripotency suggests that hESCs have the abilities to differentiate into any cell types of all three germ layers [1,2]. Biomedical researches focusing on hESCs have been lasted for nearly seventeen years since first establishment of hESC lines in 1998 [4]. However, the detailed mechanisms for self-renewal and pluripotency, which are necessary to remove the barriers for clinical application of hESC, are still obscure. Therefore, further efforts are warranted to explore and elucidate them at different levels.

Screening and identifying the key genes involved in special biological processes is one of effective ways to start hESC study and find out the reality about their growth. Several stemness genes like *Oct4*, *Nanog*, *etc*, which play important roles in regulation of pluripotency and self-renewal of hESCs, were discovered in this way [5,6]. *ESRG* (*embryonic stem cell related gene*), also known as *HESRG* (*human ESRG*), a candidate stemness gene with no homologous counterpart in other species, was first identified by us for its higher

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http://dx.doi.org/10.1016/j.bbrc.2015.02.130
0006-291X/

expression in hESCs comparing to that in 7-day embryoid bodies (EBs) by using microarray analysis [7,8]. *ESRG* expresses abundantly in undifferentiated hESC and shows a similar expression pattern to that of key stemness factors like *Oct4* in differentiated hESC which presents the possibility of ESRG as a stemness related gene in hESC. Apart from in hESC, we only detected positive ESRG expression in intracranial germinoma and embryonal carcinoma, two type cancers mainly consisted of immature and undifferentiated cancerous cells [9]. We confirmed ESRG could be served as a novel biomarker for predicting intracranial germinoma and embryonal carcinoma [9]. Furthermore, its key roles in maintenance of hESC characteristics are discovered by our group (will be published soon). However, the more precise mechanisms, like its interacting proteins which are necessary to execute its functions, still need to be further investigated.

Cytochrome *c* oxidase (COX), the last enzyme of the mitochondrial respiratory chain, comprises three mitochondria-encoded catalytic and ten nuclear-encoded regulatory subunits [10]. As a rate-limiting enzyme in electron transport chain of oxidative phosphorylation, COX plays a prominent role in energy metabolism and other related processes [11,12]. Among all the subunits of COX, COXII (cytochrome *c* oxidase subunit II) received much attentions for its significant roles in apoptosis induction, pathological processes such as ischemia, reperfusion injury and inflammation, as well as screening and prediction of degenerative diseases and cancer [13–15]. However, the functions of COXII in embryonic stem cells have been rarely studied. Here, we first identified the interaction between ESRG and COXII by yeast two-hybrid(Y2H) screening system and revealed its role in apoptosis regulation, offering an alternative mechanism to interpret apoptosis process in hESCs.

2. Materials and methods

2.1. Construction of expression vectors

pGBKT7-ESRG was constructed by linking pGBKT7, a yeast two-hybrid bait expression vector, and the open reading frame of ESRG. It was used to express a fusion protein of GAL4 DNA-binding domain (DNA-BD; amino acids 1–147) and ESRG as a bait protein. The open reading frame of ESRG was amplified from H9 cDNA by PCR with the sense primer, (5′-GAATTCCTGACTCTCTTTTCGGACTC-3′) and the antisense primer (5′- GTCGACTGAAAATAAGCGATTGGG3′).

pEF1-ESRG-*myc*-His B is an expression vector containing the open reading frame of the human ESRG gene that is tagged with C-terminal His-tag for easy detection and purification with antibodies. The open reading frame of ESRG was amplified by PCR from H9 cDNA with a couple of appropriate primers (sense primer, 5′- AGTGGATC CGCCACCATGCTGACTCTCTTTCGGACTCA–3′; antisense primer, 5′-C GAGCGGCCGCCAGGGTGAAGGAGAAGGGGTTG-3′). Then the products were cloned into *Bam*H I and *Not* I digested pEF1/*myc*-His B vector. pFLAG-CMV-3 is a 6.2 kb expression vector derived from pCMV5 and used for transient or stable expression of a properly inserted open reading frame as an N-terminal Met-FLAG fusion protein in mammalian cells. The open reading frames of AAMP, DDT, GNB2L1 were amplified by PCR from H9 cDNA with sets of appropriate primers as following and then subcloned into pFLAG-CMV-3 respectively. Each clone was entirely sequenced to confirm that no mutation was introduced by PCR. AAMP: (sense) 5′-GCGGCCGCCATGGAGTCCGAATCGG AAAG-3′, (antisense) 5′- GGATCCTTAACGGTCAGGCCTTTGGAC -3′. D DT: (sense) 5′- AAGCTTATGCCGTTCCTGGAGCTGG -3′, (antisense)5′- G GATCCTCATAAAAAAGTCATGACCG -3′. GNB2L1: (sense) 5′- AAGCTTA TGACTGAGCAGATGACCC -3′, (antisense) 5′- TCTAGACTAGCGTGTG CCAATGGTC -3′; COXII: (sense) 5′- AAGCTTATGGCACATGCAGCGCAAG -3′, (antisense) 5′- GGATCCCTATAGGGTAAATACGGGC -3′.

2.2. Yeast strain, medium, and manipulations

The yeast strain Y2Gold and Y187 (the library strains) are both conserved in the MathchMaker™ Gold Yeast two-hybrid system(-Clontech, Shiga, Japan). The yeast rich medium YPD contains 1% yeast extract, 2% peptone, 2% glucose, and 1.5% bacto agar when prepared for plates. Synthetic medium SD contains 0.67% yeast nitrogen base, 0.06% complete amino acid mixture (Clontech, Shiga, Japan), and 2% glucose. Yeast media preparation and growth were performed according to MathchMaker™ Gold Yeast two-hybrid system user manual.

2.3. Preparation of cDNA library of human ES cells

cDNA of Human ES cells (H9 cell lines) was reverse transcripted from RNA using SMART™ technology(Clontech, Shiga, Japan). The cDNA and linearized pGADT7-Rec vector were co-transformed into Yeast Strain Y187(Clontech, Shiga, Japan), which was then plated on SD/–Leu Agar medium. After 4–5 days, all colonies were pooled in rich broth medium, divided into aliquots and cryopreserved. A single 1 ml aliquot was used for each library screening.

2.4. cDNA library screening

One fresh, large (2–3 mm) colony of the bait strain was inoculated into 50 ml of SD/–Trp liquid medium and incubated by shaking (250–270 rpm) at 30 °C until the OD_{600} reaches 0.8 (16–20 h). The cells were pelleted by centrifuging (1000 g for 5 min) and resuspended in 4–5 ml SD/–Trp to a cell density of $>1 \times 10^8$ cells per ml. An aliquot (1 ml) of the library strain was thawed in a room temperature water bath, combined with 4–5 ml Bait Strain (from Step 4) in a sterile 2 L flask, and added with 45 ml of 2×YPDA liquid medium (with 50 μg/ml kanamycin). The cells from the library vial were rinsed twice with 1 ml 2×YPDA, added to the 2 L flask and incubated at 30 °C for 20–24 h with slowly shaking (30–50 rpm). After 20 h, a drop of the culture was checked under a phase contrast microscope (40×). If zygotes were not present, the cells were continuously incubateed for an additional 4 h and then pelleted by centrifuging (1000 g for 10 min). The 2 L flask was rinsed twice with 50 ml 0.5×YPDA (with 50 μg/ml kanamycin). The rinses were combined and used to resuspend the pelleted cells. Subsequently, The cells were pelleted by centrifuging (1000 g for 10 min) and discard the supernatant and resuspended in 10 ml of 0.5× YPDA/Kan liquid medium. The culture was plated with 200 μl per 150 mm on QDO (50–55 plates) and incubated at 30 °C for 3–5 days. All the colonies that grew on QDO were picked out and inoculated onto higher stringency QDO/X/A agar plates using a flat sterile toothpick or yellow pipette tip. We transformed yeast plasmid into *E. coli*, and isolated DNA isolated from *E. coli*. Then AD/library cDNA inserts can be sequenced.

2.5. Cell culture and transfection

Human ES cells from WiCell Research Institute (Madison, WI, USA) were cultured at 37 °C in a humidified incubator with 5% CO_2. The Cells were cytogenetically tested and authenticated before being frozen. The culture of hESCs were DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% knockout serum replacement (Invitrogen, Carlsbad, CA, USA), 1 mM glutamine (Invitrogen, Carlsbad, CA, USA), 0.1 mM b-mercap-toethanol (Sigma, USA), 1% nonessential amino acids (Invitrogen, Carlsbad, CA, USA), 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.

293T a cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 1% antibiotics. Plasmids were transfected into 293T cells with Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA). The cells were cultured for 36–48 h and proteins were extracted for analysis. SiRNA was transfected into human ES cells using Lipofectamine RNAiMAX (RNAiMAX) (Invitrogen, Carlsbad, CA, USA) according to our previous manual.

2.6. RNAi sequences

Double-stranded, short interfering RNA(siRNA) corresponding to COXII were designed and synthesized by RiboBio Co. (RiboBio, Guangzhou, China) are shown in Table 1.

2.7. Immunoblotting and immunoprecipitation

Protein samples were extracted with Nonidet P-40 buffer (50 mmol/L Tris-Cl, pH 8.0, 150 mmol/L NaCl, 0.5% Nonidet P-40, and protease inhibitor mixture). For immunoprecipitation, extracts were pre-cleared with 30 μl (50% slurry) agarose G beads by rocking for 2 h at 4 °C. Beads were removed, 30 μl (50% slurry) of fresh agarose G beads and appropriate antibodies were added to the pre-cleared lysates, and the mixture were incubated overnight at 4 °C. The beads were washed, mixed with 2 × SDS sample buffer, boiled, and then resolved by 12% SDS–PAGE. Proteins were detected with specific antibodies and an ALP–conjugated secondary anti-body.

2.8. Western blot analysis

Western blot was carried out as previously described with a minor modification [8,16]. Mouse anti 6 × His-Tag monoclonal antibody, rabbit anti 6 × His-Tag polyclonal antibody, rabbit anti-bcl2 antibody, rabbit anti-Bcl-xL antibody, rabbit anti-Bax antibody, rabbit anti-PARP antibody, rabbit anti-cleave-PARP antibody(c-PARP), rabbit anti-Apaf-1 antibody, rabbit anti-Cytochrome *c* antibody and Caspase 3 were purchased from Proteintech (Proteintech, Wuhan, China). Rabbit anti MT-COXII(COXII) polyclonal antibody was purchased from Sangon (Sangon Antibody R&D Center, Shanghai, China). Anti-mouse IgG antibody was purchased from Abcam (Cambridge, MA, USA). Anti-mouse β-actin, anti-Flag, goat anti-rabbit poly-HRP and anti-mouse poly-HRP were purchased from Sigma (Rockford, USA). Rabbit anti Flag-Tag polyclonal antibody was purchased from CST (Danvers, Massachusetts, USA).

2.9. Statistical analysis

Statistical analyses were performed using SPSS version 11.5 statistical software (USA). The paired Student t-test was adopted to determine statistical significance. The statistical significance level was set at $p < 0.05$.

3. Results

3.1. No autonomous activation and toxicity was detected in the Y2H system

To guarantee the effectiveness of the Y2H system, it is imperative to exclude the existence of autonomous activation and toxicity in the absence of prey protein [17]. Here, pGBKT7-ESRG-Y2HGold can proliferate on SD/-Trp plate (Fig. 1A, left panel) but not on SD/–Ade/–His/–Leu/–Trp/X-α-Gal/Aba (Fig. 1A, right panel) plate which eliminated the possibility of auto-activation. At the same time, no significant difference of proliferation rate demonstrated by the size of clones, was observed between pGBKT7-ESRG-Y2HGold and pGBKT7-Y2HGold yeasts, excluding the deleterious possibility of ESRG to yeast host (Fig. 1B). The above results suggested that the Y2H system was reliable and could be applied in the following procedures.

3.2. Four candidate ESRG-interacting proteins were identified

At the beginning, the quantified titer of H9 cDNA library was validated by gradient dilution (Fig. 1C, D). Subsequently, 29 and 18 candidate positive clones with blue color were observed on SD/-His/-Leu/-Trp/-Ade/X-α-Gal/Aba plates respectively in two independent screenings (Fig. 1E, F). After being amplified in SD/-Leu/-Trp liquid medium, the plasmids were extracted and the target cDNA was sequenced. By sequence alignment with BLAST software of NCBI (Table 2), we obtained four candidate ESRG-interacting proteins, including AAMP (Angio-Associated Migratory Cell Protein), DDT (D-Dopachrome Tautomerase), GNB2L1 (Guanine Nucleotide Binding Protein (G Protein), Beta Polypeptide 2-Like 1), and COXII (Cytochrome *C* Oxidase Subunit II).

3.3. Interaction between ESRG and COXII was confirmed by Co-IP

Subsequently, Co-IP was carried out to verify the interactions between ESRG and candidate proteins. Mammalian expression plasmid encoding His-ESRG(ORF) fusion protein was co-transfected into HEK-293T cells with plasmids encoding Flag-AAPM, Flag-DDT, Flag-GNB2L1, or Flag-COXII respectively. The expression of corresponding proteins was detected by Western blot with His or Flag monoclonal antibodies to confirm the successful trsanfection (Fig. 2A, B, C). The cell lysates were prepared and immunoprecipitated with anti-Flag antibody, then anti-His was used to detect the existence of His-ESRG in the immunoprecipitation complex. As shown in Fig. 2A, B, C, no His tag was detected in immunoprecipitation complex by anti-His antibody. However, the interaction between COXII and ESRG was observed in 293T cells(Fig. 2D,E). In addition, the ESRG and COXII interaction was observed in H9 cells (Fig. 2F). The above results confirmed the interaction between ESRG and COXII.

Table 1
Sequences for designed si-hCOXII.

Number	Target mRNA sequences	Sense sequences	Antisense sequences
si-h-COXII-1	CGTTGACAATCGAGTAGTA	5′-CGUUGACAAUCGAGUAGUAdTdT-3′	3′-dTdTGCAACUGUUAGCUCAUCAU-5′
si-h-COXII-2	CCATCGTCCTAGAATTAAT	5′-CCAUCGUCCUAGAAUUAAUdTdT-3′	3′-dTdTGGUAGCAGGAUCUUAAUUA-5′
si-h-COXII-3	AGCGCAAGTAGGTCTACAA	5′-AGCGCAAGUAGGUCUACAAdTdT-3′	3′-dTdTUCGCGUUCAUCCAGAUGUU-5′
si-h-COXII-4	GGAGCAAACCACAGTTTCA	5′-GGAGCAAACCACAGUUUCAdTdT-3′	3′-dTdTCCUCGUUUGGUGUCAAAGU-5′
si-h-COXII-5	CTACGCATCCTTTACATAA	5′-CUACGCAUCCUUUACAUAA dTdT-3′	3′-dTdTGAUGCGUAGGAAAUGUAUU-5′

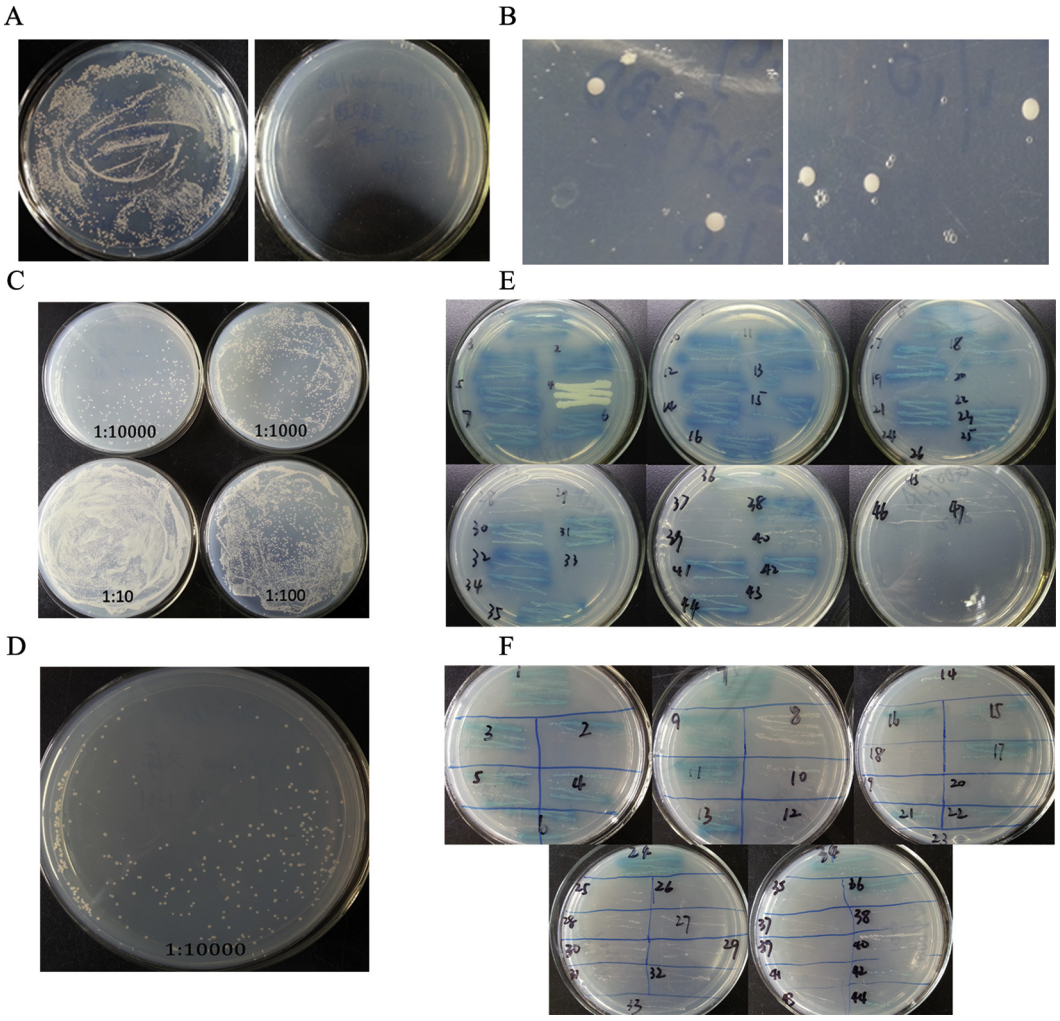

Fig. 1. Autonomous activation and toxicity assay of Y2H system and screening the interacting proteins of ESRG. No autonomous activation was detected for pGBKT7-ESRG-Y2HGold can proliferate on SD/-Trp plate (A, left panel) but not on SD/−Ade/−His/−Leu/−Trp/X-a-Gal/AbA plate (A, right panel). The toxicity of ESRG was excluded by the similar sizes of clones for pGBKT7-ESRG-Y2HGold (B, left panel) and pGBKT7-Y2HGold (B, right panel). The titer of H9 cDNA library was counted by gradient dilution (C) and the qualified titer was achieved, reflected by more than 200 colonies on 1/10000 dilution plate (D). 28 clones among 47 ones (E) and 18 clones among 44 ones (F) were stained by blue color in two independent screenings. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Downregulation of COXII expression protects hESCs from mitochondria related apoptosis

To investigate the role of COXII in hESCs, 5 targeted small interfering RNA sequences (Table 1) were designed to down-regulate the expression of COXII. The si-RNA sequences were transfected into H9 cells respectively. Two days later, the cells were collected for total RNA,and collected protein extraction after three days to detect the interference efficiency by RT-PCR and Western blot, respectively. The maximal knockdown efficiency was achieved for the sequences 2 and 5 (Fig. 3), which were used for the following functional study.

Table 2
The BLAST results of positive clones.

Protein no.	Protein name	Gene	NCBI protein accession no. Human	Max identity (%)
1	Angio-Associated Migratory Cell Protein	AAMP	CAG33036	99
2	D-Dopachrome Tautomerase	DDT	CAG30317	99
3	Guanine Nucleotide Binding Protein (G Protein), Beta Polypeptide 2-Like 1	GNB2L1	CAG33259	100
4	Cytochrome C Oxidase Subunit II	COXII	ACN37834	97

As mentioned above, aberrant expression of COXII is involved in multiple physiological or pathological processes [11,14]. Here, we mainly assessed its influence on mitochondria related apoptosis in hESCs. The lysates of H9 cells and H9 cells transfected with si-COXII-2 (si-COXII-2-H9) or si-COXII-5 (si-COXII-5-H9) were prepared for expression detection of apoptosis-related proteins by Western blot. First, the expression of pro-apoptosis proteins was assayed. Obviously, the expressions of p53, Bax, Apaf-1 and Cytochrome *c* were all downregulated in both si-COXII-2-H9 and si-COXII-5-H9 cells, compared to those in H9 cells (Fig. 4A, B). There were no significant differences in expressions of Caspase3, PARP and cleaved PARP between H9 and si-COXII-2-H9 or si-COXII-5-H9 cells (Fig. 4C). Then, the expressions of anti-apoptosis proteins, like Bcl-xL, Bcl-2, and Survivin, were analyzed and they all were upregulated in si-COXII-2-H9 or si-COXII-5-H9 cells (Fig. 4D). It turned out that reduced COXII expression significantly increased the expressions of these proteins. These corresponding expression alterations of pro- and anti-apoptosis proteins revealed that COXII may serve as a positive regulator in mitochondria related apoptosis in hESCs.

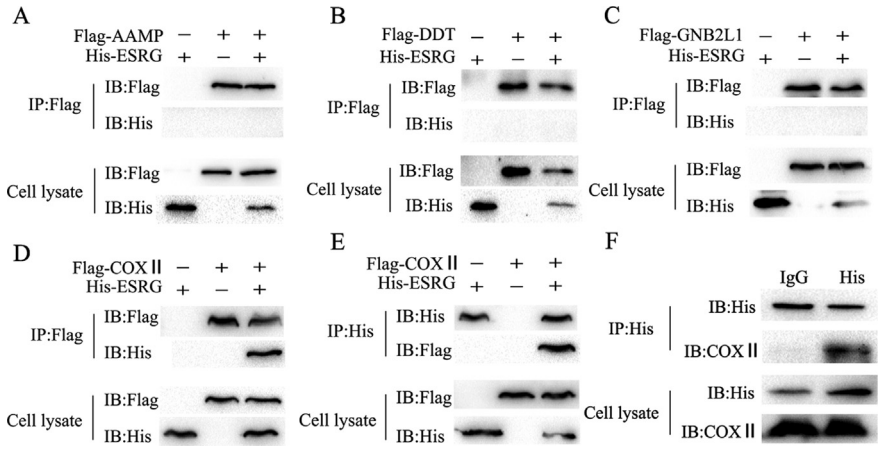

Fig. 2. Identifying the interactions between ESRG and candidate proteins by Co-IP. No His-ESRG bands were detected in complexes immunoprecipitated by anti-Flag in Flag-AAPM, Flag-DDT or Flag-GNB2L1 transfected 293T cells, respectively (A,B,C). However, the interaction between ESRG and COXII was confirmed. Flag-COXII and His-ESRG were cotransfected into 239T cells. At 36 h after transfection, cell lysates were harvested and Flag- COXII or His-ESRG was immunoprecipitated with anti-Flag or anti-His. Western blotting was performed(D, E). The ESRG and COXII interaction was observed in H9 cells (F). Cell extracts were immunoprecipitated with anti-Flag or control IgG. The immunoprecipitated complex was detected by Western blotting with a COXII antibody.

4. Discussion

Thus far, tremendous efforts have been devoted to the studies of embryonic stem cells for its promising potential in clinical applications which would exert revolutionary influences on human health management [1,2]. The previous work, especially for the success of transforming the somatic cells into induced pluripotency stem cells by ectopic expressions of transcriptional factors in multiple combination, has demonstrated the key regulated roles of stemness genes in maintaining the characteristics of embryonic stem cells [5,6,18]. ESRG was first cloned by our group and its subcellular localization, different splicing transcripts and expression patterns in hESCs and other tissues had already been identified [7,8]. Furthermore, its key roles in supporting the characteristics of hESCs had been revealed by our unpublished work (would be published soon). In this paper, we looked forward to identifying ESRG-interacting proteins in hESCs by yeast two-hybrid system, in order to clarify the role of ESRG in characteristics maintenance of hESCs and elucidating its relative position in the regulated networks of hESCs.

Since first invented by Fields S and Song O in 1989, the yeast two-hybrid system has contributed a lot to understanding the molecular landscape of cells [19]. With successive refinement and modification, it has been widely applied in almost every field of biological and medical researches, especially in interpreting the cell signaling network and medicine screening [17,20–22]. In this work, yeast two-hybrid system was adopted to find ESRG-interacting proteins in hESCs by using ESRG as the bait protein. The absence of auto-activation and self-toxicity effect was confirmed at first to guarantee the effectiveness of screening. Four candidate proteins, AAMP, DDT, GNB2L1 and COXII, were screened after amplification, sequencing and alignment. Considering the possibility of false positive of yeast two-hybrid results due to technical defects and biological factors, further procedures must be performed to validate the protein–protein interactions (PPIs), and co-immunoprecipitation is the prior and direct choice to verify PPIs. The interactions between ESRG and candidate proteins were

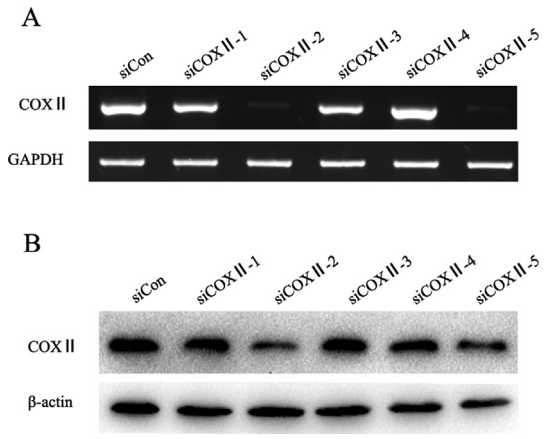

Fig. 3. Knockdown the expression of COXII in H9 cells. Five si-RNA sequences targeting COXII were transfected into H9 cells, and the best knockdown efficacy was achieved by sequence 2 and 5 demonstrated by RT-PCR (A) and WB (B).

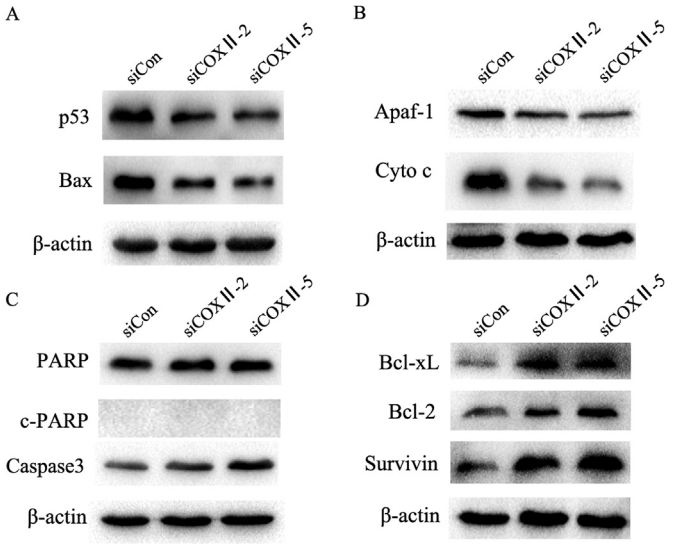

Fig. 4. Apoptosis analysis of H9 cells with inhibited COXII expression. Apparently, the mitochondrial apoptosis signaling was suppressed exhibited by: downregulated expressions of pro-apoptotic proteins like p53 and Bax (A), Apaf-1 and Cyto *c* (B); comparable expression of Caspase-3., PARP, c-PARP(C); upregulated expressions of anti-apoptotic proteins like Bcl-xL, Bcl-2 and Survivin (D) in H9 cells with depressed COXII level.

analyzed in 293T cells with the corresponding anti-tag (His or Flag). Positive signals were only observed between ESRG and COXII. Additional work was necessary to be done to clarify the key domains, intracellular location, as well as the function of the interaction.

The pro-apoptosis role of COXII had been generally confirmed in a cohort of studies [23,24]. The up-regulation of COXII was an early apoptotic incident in different types of cells, including T cells and cancer cells, induced by HIV infection or agents [15,25]. It seemed to serve as a "switch" of apoptosis for some proteins can interact with it and then initiate the apoptosis process, which may due to its high affinity to Cytochrome *c* [24,26]. However, its roles in hESC were not explored yet. Here, COXII exerted a similar pro-apoptosis role in hESCs reflected by the down-regulation positive regulators like p53, Bax, Apaf1, Cyto *c*, and the up-regulation of negative regulators such as Bcl-xL, Bcl-2 and Survivin in si-COXII-H9 cells.

In conclusion, we initially identified the interaction between ESRG and COXII by Y2H system and Co-IP. The pro-apoptotic role of COXII in hESCs was also validated by knocking down its expression. Probably, ESRG could take part in the regulation of apoptosis of hESCs by interaction with COXII, which may demonstrate an alternative apoptosis path in hESCs.

Conflict of interest

None declared.

Acknowledgments

This work was supported by National Natural Science Foundation of China (30871246, 81070993), Specialized Research Fund for the Doctoral Program of Higher Education of China (SRFDP) (20120162110059), National Basic Research Program of China (2010CB833605), Program for New Century Excellent Talents in University (NCET-10-0790), Hunan Provincial Innovation Foundation for Postgraduate (CX2012B081), Hunan Provincial Science and Technology Department (2011FJ4180), Open-End Fund for the Valuable and Precision Instruments of Central South University.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.130.

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