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Construction and identification of mouse R-spondin 1 eukaryotic expression vector

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Abstract

R-spondin1 (Rspo1) has been proposed to activate and synergize with the Wnt signaling pathway. It is involved in pancreas and muscle development, bone formation, limb and gonad morphogenesis, even associated with many human diseases. In this study, we amplified the full-length Rspo1 gene from mouse pancreas using RT-PCR technology. The recombinant pMD19-T-Rspo1 vector was constructed by connecting pMD19-T vector and full-length Rspo1 gene, and then the Rspo1 full-length fragment sequenced correctly was connected to pIRES2-AcGFP1 vector after double digestion with *Eco*R I and *Bam*H I. The recombinant vector pRspo1-IRES2-AcGFP1 was identified by double digestion of *Eco*R I and *Bam*H I. We examined the expression of green fluorescent protein (GFP) under fluorescent microscope and the mRNA expression level of Rspo1 using RT-PCR after transfection it into HEK293 cells for 48 h. These results indicated that Rspo1 eukaryotic expression vector had been constructed successfully and identified. This would lay the foundation for further research on regulatory function of Rspo1 on Wnt signaling pathways and mechanism on proliferation and differentiation of stem cells.

Key words: R-spondin1 (Rspo1) , pRspo1-IRES2-AcGFP1, HEK293 cells, eukaryotic expression vector.

Introduction

Wnt signaling plays important roles in development as well as in the pathogenesis of a variety of diseases, including diabetes [1-3]. Activation of this pathway requires interaction between a secreted glycoprotein, Wnt, and a seven transmembrane receptor protein, Frizzled (Frz). There are at least three distinct intracellular Wnt pathways, including, most notably, the canonical Wnt (cWnt) cascade that leads to changes in intracellular β-catenin expression levels. It is thought to be involved in cell fate specification and proliferation. β-catenin is normally phosphorylated and targeted for proteolysis by a complex of proteins, including adenomatosis polyposis coli (APC), axin and the serine/threonine kinase glycogen synthase kinase-3 β (GSK3 β). cWnt activation of the Frz and low density lipoprotein receptor-related protein (LRP) co-receptors results in dissociation of this degradation complex, permitting entry of β -catenin into the nucleus to activate cWnt target genes in conjunction with TCF/LEF family transcription factors and, possibly, other DNA-binding partners [4]. cWnt target genes have been identified in different models and these include, but are not limited to, the cell-cycling genes, c-myc, and cyclin D1 [5].

The roof plate-specific spondin (R-spondin, Rspo) protein family consists of four structurally related members (Rspo14), which are recently described, with conserved cysteine-rich furin-like and thrombospondin domains [6,7]. Several evidences indicate that Rspo family members function as Frz and/or LRP receptor ligands *in vitro*: 1) Rspo is a secreted protein [8]; 2) unlike Wnt ligands that form a ternary complex with Frz and LRP receptors, Rspo proteins failed to form a ternary complex but can nonetheless bind to both receptors [9]; 3) there is a positive modulation of Wnt ligand activity by Rspo via direct interaction between the two ligands [10, 11]; and 4) Rspo prevents LRP6 internalization [12, 13]. Furthermore, transgenic mice expressing human Rspo1 exhibits a profound increase in proliferation of intestinal crypt epithelial cells, which correlates with the activation of β-catenin [8]. Adenoviral-mediated transfection of each isoform of Rspo into mice also induces gastrointestinal proliferation in association with β-catenin activation [7].

To date, although multiple function and regulatory mechanism of Rspo1 have been clarified, its roles in proliferation and differentiation of pancreatic stem cells keep still unclear. In our previous study, we found that activation of Wnt/β-catenin pathway can robustly stimulate pancreatic proliferation and mass formation [14]. In the present study, we will construct Rspo1 eukaryotic expression vector and transfect it into HEK 293 cell line for future exploring the mechanism of Rspo1 in regulating pancreatic stem cell.

Materials and Methods

Plasmid, host cell and cell line: Eukaryotic expression vector pIRES2-AcGFP1 and HEK293 cell line were kept by Shaanxi Stem Cell Engineering and Technology Research Center. HEK293 cells were cultured in DMEM with 10% fetal bovine serum. The vector pMD19-T and DH5α were purchased form TaKaRa (Dalian, China).

RNA extraction and semi-quantitative RT-PCR: Total RNA was extracted from different tissues and cells using TRIzol Reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was dissolved in 20 µl nuclease-free water. RT-PCR was performed according to a coupled one-step procedure using the Access RT-PCR System (Promega, Madison, WI, USA). Briefly, 2 µg of total RNA was reverse transcribed at 37°C for 1 h, denatured at 94°C for 2 min and amplified for 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec and extension at 72°C for 45 sec, with a final extension step of 10 min at 72°C. The amplified products were analyzed by electrophoresis on a 1% agarose gel. Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a High Performance CCD camera. β-actin was used as an internal control. The sequence of specific primers was designed according to the sequence of Rspo1 gene (Genebank No.: NM_138683.2). And the primers were as follows: F: 5'-CCGG AATTCCTATGCGGCTTGGGCTGTGC-3'; R: 5'-CGC GGATCCCGGTCACTGTGCCCAGGTAGG -3'. The underlined bases were the recognition sites of *Eco*R I and *Bam*H I restriction enzymes, respectively. Several protective bases were designed ahead of the recognition sites.

Construction of p Rspo1-IRES2-AcGFP1 vector: The full-length Rspo1 fragment was obtained from mouse pancreas using PCR amplification. The product of PCR was connected with pMD19-T vector with T4 ligase (TaKaRa, Dalian, China) and transformed DH5α competent cells. Then the plamid were extracted and identified with double digestion of EcoR I/BamH I. Some samples were sent to Sangon Biotech Co., Ltd (Shanghai, China) for sequencing. We named plasmid sequenced correctly as pMD19-T-Rspo1. Then pMD19-T-Rspo1 and pIRES2-AcGFP1 vector were double digested with *Eco*R I/*Bam*H I. The target fragment was connected with T4 ligase (TaKaRa, Dalian, China). Then the product of ligation was identified with double digestion of *Eco*R I/*Bam*H I. We named expression vectors identified correctly as pRspo1-IRES2-AcGFP1 and was used to the following experiments.

Cell transfection: Transient transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol with minor modification. The concrete steps were as follows. HEK293 cells were passaged and plated at a concentration of 2×10^5 per well of 6-well plate one day before transfection. One day later, transfection protocol began when cell reached 80% confluence. The medium was changed with 1 ml OptiMEM per well. Then we diluted 2 µg DNA of pIRES2-AcGFP1 or pRspo1-IRES2-AcGFP1 into 250 µl OptiMEM medium and 10 µl Lipofectamin 2000 into 250 µl OptiMEM medium. After incubation for 5 min at room temperature, we combined it with the DNA and incubated another 30 min. Add 250 µl of the diluted Lipofectamin 2000 to each well containing diluted DNA. They were mixed gently and incubated at room temperature for 20 min to allow DNA-Lipofectamin 2000 complexes to form. The DNA-Lipofectamine 2000 complexes (500 µl) was added directly to each well of the plates containing cells and mixed gently.

Results and Discussion

Amplification of Rspo1 full-length fragment: We extracted RNA and amplified Rspo1 full-length fragment from mouse brain, pancreas, small intestine and testis. As shown in Fig. 1, we detected obvious mRNA expression of Rspo1 in pancreas (Fig. 1). The size of fragment was approximately 800 bp (Fig. 1). This could be used to construct its eukaryotic expression vector. At the same time, we found that the mRNA expression level of Rspo1 was variant in different mouse tissues. This might indicate that Rspo1 exert different roles on various tissues.

Identification of recombinant vector pMD19-T-Rspo1: To identify the correctness of pMD19-T-Rspo1 vector, we picked 12 clones to evaluate after connecting pMD19-T and Rspo1 fragment. Fortunately, as seen in Fig. 2, we observed two electrophoretic brands after being cut by *Eco*R I and *Bam*H I in all 12 samples (Fig. 2). The sizes of two brands were approximately 800 bp and over 2000 bp (Fig. 2). These were same as the expected ones. Then two corresponding bacterial fluid of samples were sent to sequence.

Construction and identification of pRspo1-IRES2-AcGFP1 expression vector: To construct pRspo1-IRES2-AcGFP1 expression vector, we digest pIRES2-AcGFP1 and pMD19-Rspo1 sequenced correctly using two restriction enzymes of *Eco*R I and *Bam*H I. As shown in Fig. 3, we obtained two target fragments of Rspo1 and IRES2-AcGFP1 (Fig. 3). The sizes were approximately 800 bp and 5000 bp, respectively (Fig. 3). After ligation, we transformed the ligation products into competence cells and extracted the plasmids for identifying the accuracy of recombinant vector using double digestion. We could observe clearly two electrophoretic bands with corresponding sizes among 11 selected samples at random (Fig. 4). These plasmids identified correctly were names as pRspo1-IRES2-AcGFP1.

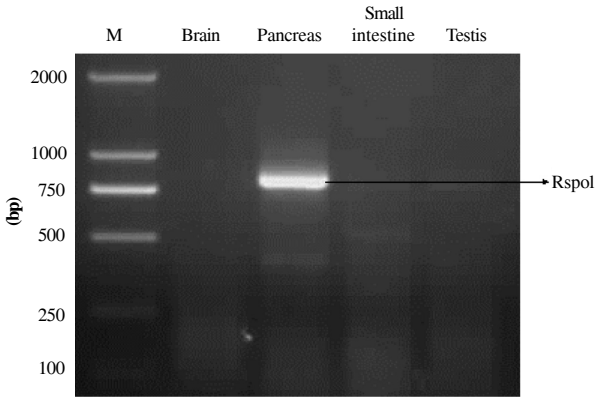

Figure 1. The electrophoresis result of amplifying Rspo1 gene from different mouse tissues using PCR assay. M: DL2000.

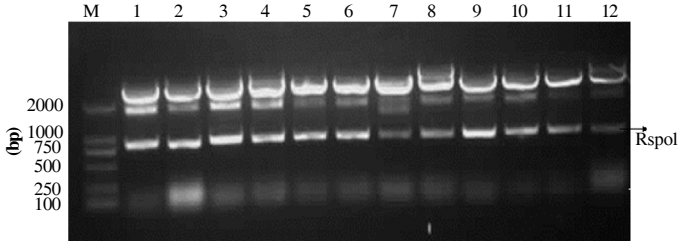

Figure 2. Double digestion identification of pMD19-T-Rspo1 using *Eco*R I and *Bam*H I. M: DL2000, Lane 1-12: Different clones.

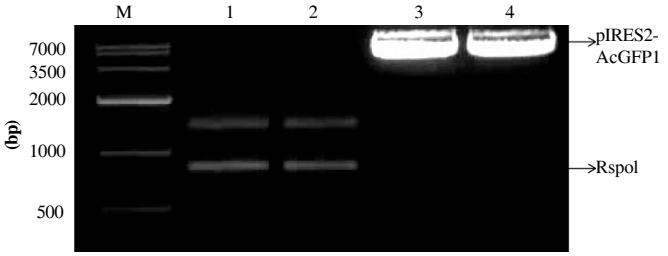

Figure 3. Double digestion of pIRES2-AcGFP1 and pMD19-Rspo1 using *Eco*R I and *Bam*H I. M: Marker IV; Lane 1, 2: Double digestion of pMD19-Rspo1; Lane 3, 4: Double digestion of pIRES2-AcGFP1.

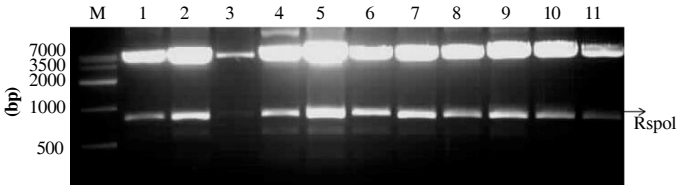

Figure 4. Identification of pRspo1-IRES2-AcGFP1 using double digestion with *Eco*R I and *Bam*H I. Lane 1-12: Different clones.

Green fluorescent protein expression in HEK293 cells: We observed the obvious expression of green fluorescent protein (GFP) in HEK293 cells after transfecting pRspo1-IRES2-AcGFP1 for 48 h (Fig. 5). The efficiency of transfection was approximately 70-80% by counting the numbers of cells under visible light and fluorescent microscope at the same field of view (Fig. 5). This result might imply that the pRspo1-IRES2-AcGFP1 can act as a tool in future studying the expression and function of Rspo1.

R-spo1 expression in HEK293 cells after transient transfection: To assure the expression of pRspo1-IRES2-AcGFP1, we detected the mRNA expression of Rspo1 in HEK293 cells after transfecting this expression vector for 48 h. The results indicated that the mRNA expression of Rspo1 was very strong in transfecting pRspo1-IRES2-AcGFP1 group, however, no Rspo1 expression was observed in transfecting empty group (Fig. 6). This might imply that Rspo1 eukaryotic expression vector was constructed successfully and could be expressed in eukaryotic cells.

Rspo1 was shown to have a mitogenic influence on intestinal epithelium [8] and to be essential in human sex determination [15]. In mice, inactivation of Rspo1 by homologous recombination resulted in the appearance of XX partially sex-reversed mice [16, 17]. These observations are consistent with the reported sexually dimorphic expression of this gene in embryonic mouse gonadic somatic cells[15, 16] and its implication in the activation of Wnt-4 and β-

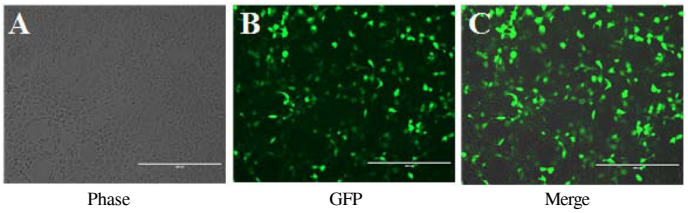

Figure 5. The expression of green fluorescent protein (GFP) after being transfected with pRspo1-IRES2-AcGFP1 in HEK 293. A: The transfected HEK293 cells under visible light; B: The transfected HEK293 cells under fluorescent microscope. C: The overlap of A and B. (bar = 400 μm)

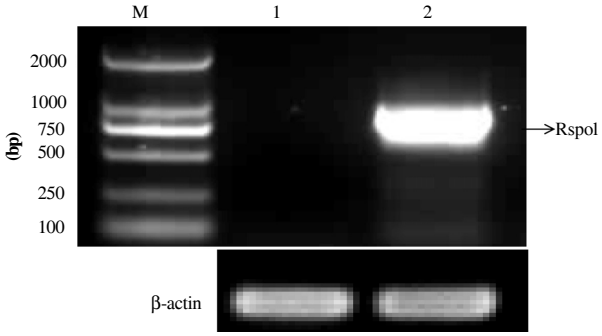

Figure 6. The mRNA expression of Rspo1 after being transfected pRspo1-IRES2-AcGFP1 in HEK293. M: DNA marker (DL 2000). Lane 1: pIRES2-AcGFP1 transfection group; 2: pRspo1-IRES2-AcGFP1 transfection group.

catenin pathways [17]. Comparative expression studies of Rspo1 in various vertebrate species suggested that this gene, that encodes for an evolutionary highly conserved protein, has a preserved role in ovarian development [18, 19]. Although, diverse roles of Rspo1 have been explored, its function in pancreatic stem cells was still not mentioned. At present, the difficulties of pancreatic stem cell exiting in the utilization and study are the low proliferative and insulin-secreting ability. We have found that activation of Wnt/β-catenin pathway could increase the proliferative ability of pancreatic stem cell. In order to further explore and amplify the roles of Wnt singling pathway on pancreatic stem cell, we construct successfully Rpo1 eukaryotic expression vector with GFP gene. We could detect the expression of Rspo1 in a way of real-time comprehensive observation in a dynamics. At the same time, we could collect the conditioned medium used for treatment pancreatic stem cells after tansfecting the pRspo1-IRES2-AcGFP1 into HEK 293 cells.

Conclusions

In this study, we obtained the full-length fragment of Rspo1 from mouse pancreas using RT-PCR assay, and then linked the fragment identified correctly with pIRES2-AcGFP1 after double digestion. Ultimately, eukaryotic expression vector of Rspo1-IRES2-AcGFP1 was constructed successfully. This would lay the foundation on the further study on the function and mechanism of Rspo1 in proliferation and differentiation of stem cells.

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