Establishment and Characterization of Inducible Cell Line with Stable Expression of Spata3

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Abstract *Spata3* is a testis-specific expression gene and may play a role in spermatogenesis or spermatogenesis cell apoptosis. To further investigate the spata3 function, we successfully established the cell line which can stably express Flp-In™T-REx™-spata3 under the induction of tetractcline or doxycline by cloning the *spata3* ORF into our modified pcDNA5/FRT/TO expression vector, with the Flp-In™T-REx™-293 cell line as a host line. This cell line has 2×FLAG tag and 2×His tag in the 3'- prime of *spata3* and the full-length protein of spata3 can therefore be easily detected by Western blotting with FLAG tag and/or His tag antibody. This procedure is not only very useful for *spata3* functional study, but also a useful reference for other protein complex(s) purification and function identification by using highly specific anti-FLAG antibody.

Key words *spata3*; stable cell line; protein expression; induction

Functional study for protein and/or protein complex (s) requires its expression efficiently in mammal cells. The cell behaviors conducted by proteins and characterization of proteins requires their stable expression in mammal cells. High-efficiency tools for establishing inducible cell lines will fit our requirement in general. The *spata3* (former named *Mtsarg1*, AY032925) is a testis-specific expression gene that was cloned by bioinformatics and experience and may be related to spermatogenesis or spermatogenesis cell apoptosis[1]. So far, there still don't have much *spata3* functional study and available specific antibody for spata3. To further investigate the spata3 function, and to use *spata3* as example for establishing stably inducible cell line, we successfully established the cell line which can stably express Flp-In™T-REx™-spata3 under the induction of tetracycline or doxycline (DOX) by cloning the *spata3* ORF into our modified pcDNA5/FRT/TO expression vector. This technique and procedure also is the very useful reference for other protein complex(s) purification and function identification with the use of highly affinitive and specific anti-FLAG antibody.

1 Materials and Methods

1.1 Nested RT-PCR amplification

The mouse testis total RNA was made according the literature and using the primer P3 (5'-ccggacggtg-ggcctggggtc-3') performed reverse transcript and finished two steps nested PCR to amplify the ORF of *spata3* according to the literature [1]. The amplified DNA was digested by the *Nhe*I at the 3'-primes.

1.2 The modified pcDNA5/FRT/TO-*spata3* construct

The pcDNA5/FRT/TO was purchased from invitrogen (Catalog no. V6520-20) and cloned the 2×FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and 2×His tag (His-His-His-His-His-His) at the 3'-prime of polyclone site as a modified pcDNA5/FRT/TO vector. Then modified vector was digested by *Eco*RV and *Nhe*I, and ligated with digested *spata3*, and constructed the modified pcDNA5/FRT/TO-*spata3* construct. The recombinant construct was verified by PCR and sequencing.

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1.3 Generation of stable expression cell line

Flp-In™T-REx™-293 cell line (Catalog no. K6500-01, Invitrogen) was used to generate the cell line with Flp-In™T-REx™-spata3 under the induction of tetracycline. The modified pcDNA5/FRT/TO-*spata3* construct and pOG44 plasmid, which expresses Flp recombinase was cotransfected into host cell line in six-well plates. The next day split the cell into the 10 cm plat.

1.4 Polyclonal selection of isogenic cell lines

Stable transfectants were selected using hygromycin B (Invitrogen). To obtain stable expression cell lines, polyclonal selection and screening of our hygromycin-resistant cells were performed. After hygromycin killed the negative cells, the hygromycin-resistant foci were pooled.

1.5 Induction of spata3 expression

Once we generated the 293 cell line with Flp-In™T-REx™-spata3, 0.5 μg/ml final concentration DOX was added, and incubated the cells for 1 d at 37 ℃ before harvesting. Then, in order to test the dosage effect, different amount of DOX was added and the cells were incubated for 1 d at 37 ℃. The protein whole cell lysates (WCL) were made with EBC buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1×protease inhibitor cocktail].

1.6 Western blotting analysis [2,3]

The samples were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membrane was incubated with blocking buffer (50 mmol/L Tris-HCl/150 mmol/L NaCl containing 5% milk) 1 h. The primary antibodies (anti-FLAG M2 antibody, Sigma) were diluted in incubating buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 7.5), 0.1% Tween 20, 2% milk] and added to the membranes for overnight rotation at 4 ℃. The membrane was washed 3 times, each 15 mins with 1×TBST washing buffer (50 mmol/L Tris-HCl/150 mmol/L NaCl/0.05%Tween-20). The membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Then the membrane was washed 3 times, each 10 mins with 1×TBST washing buffer [2,3]. All blots were visualized with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill.) and photographed.

2 Results

2.1 Construction of the modified pcDNA5/FRT/TO-*spata3* construct

The pcDNA5/FRT/TO was purchased from invitrogen and cloned the 2×FLAG and 2×His tag at the 3'- prime of polyclone site as a modified pcDNA5/FRT/TO vector. The modified vector was cut with *Eco*RV and *Nhe*I, ligated it with same enzymes digested spata3 by nested RT-PCR technique from mouse testis tissue, and finally cloned the modified pcDNA5/FRT/TO-*spata3* construct. The recombinant construct was verified with PCR amplification (Fig.1) and DNA sequencing.

2.2 Generation of stable expression *spata3* cell line

The Flp-In™T-REx™-293 cell line was used as a host cell line. Flp recombinase will mediate insertion of our pcDNA5/FRT/TO-*spata3* expression construct into the genome at the integrated FRT site through site-specific DNA recombination [4,5]. After hygromycin selection, we simply pooled the hygromycin-resistant foci. Finally stable expression *spata3* cell line was generated.

2.3 Induction of spata3

In order to detect the spata3 expression, we lysated the cells with or without DOX induction or host cell line. The Western blotting was performed by anti FLAG M2 antibody. Fig.2 showed that after induction, an about 31 kDa protein band was detected, and without induction or host cell line only cannot be detected any signal, α-tubulin as a loading control, which showed that we have generated the inducible cell line with stable expression of spata3.

2.4 The dosage of DOX for induction of *spata3*

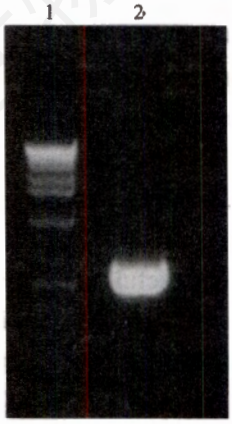

Fig.1 PCR verified the modified pcDNA5/FRT/TO-*spata3* insert

1: 1 kb ladder; 2: *spata3*.

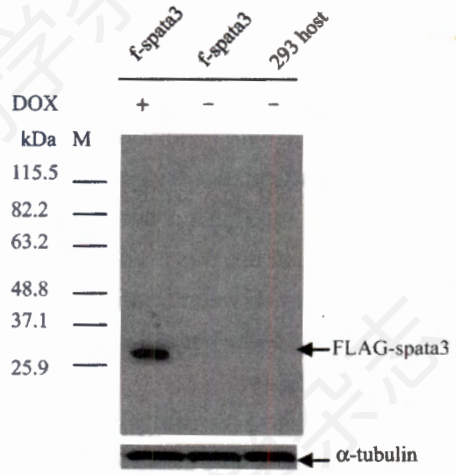

Fig.2 Spata3 expression was induced by DOX in spata3 inducible expression stable cell line

M indicates the protein molecular weight. (–) showed without DOX. α-tubulin as a control showed the equal amount of total protein loading.

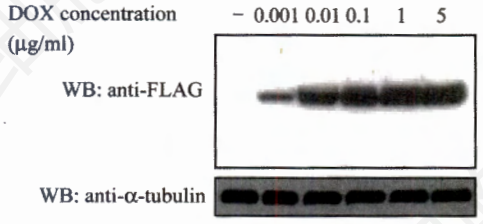

Fig.3 The spata3 expression level by different amount of DOX

(–) showed without DOX; α-tubulin as a control.

To test the dosage effect, different amount of DOX (Fig.3) was added and incubated the cells for 1 d at 37 ℃ before harvesting. The protein WCL was made and Western blotting was performed. Comparing with the loading control α-tubulin, the concentration from 0.001 μg/ml to 5 μg/ml DOX, all showed the spata3 expression, but 0.001 μg/ml showed very weak expression and 1 to 5 μg/ml showed similar higher expression. The expression level from 0.001 to 1 μg/ml was gradually increased. So, protein expression level can be modulated and we chose the concentration 0.1–1 μg/ml of DOX for protein expression induction in general.

3 Discussion

There are some advantages of the modified pcDNA5/FRT/TO-*spata3* construct system. First, pcDNA5/FRT/TO is an inducible expression vector designed by using the system with the Flp-In™T-REx™. The vector contains the following elements: A hybrid human cytomegalovirus (CMV)/*Tet*O2 promoter for high-level, tetracycline/DOX regulated expression of the gene of interest in a wide range of mammalian cells [6~9]; Multiple cloning site with unique restriction sites to facilitate cloning one copy of the gene into FLP recombination target (FRT) site for Flp recombinase-mediated integration of the vector into the Flp-In™T-REx™ host cell; Hygromycin resistance gene for selection of stable cell lines [10]. Expression of our target gene *spata3* is controlled by the strong CMV immediate early enhancer/promoter [6,7] into which 2 copies of the *tet* operator 2 (*Tet*O2) sequence have been inserted in tandem. Insertion of these *Tet*O2 sequences into the CMV promoter confers regulation by tetracycline to the promoter. We use the Flp-In™T-REx™-293 cell line as a host to generate a tetracycline-inducible Flp-In™T-REx™ expression cell line by cotransfecting the pcDNA5/FRT/TO expression vector containing *spata3* and the Flp recombinase expression plasmid, pOG44[4]. Flp recombinase mediates insertion of one copy of our pcDNA5/FRT/TO expression construct into the genome at the integrated FRT site through site-specific DNA [4,5]. Once a stable cell line has been generated, expression of our target gene can be induced with tetracycline or DOX. We usually use the DOX to induce the expression because DOX has a longer half-life time (2 days) than tetracycline (one day). Second, the inducible expression level can be regulated by added different amount of DOX or tertracycline (Fig.3). Third, important of all, we modified the pcDNA5/FRT/TO vector that has the 2×FLAG and 2×His tag at the 3'- prime of polyclone site. We can easily detect full-length protein by using anti-FLAG or anti-His antibody to perform Western blotting, IP or protein complex(s) purification while the spata3 antibody unavailable. Especially, it also is very useful for protein complex(s) purification to investigate the protein function and protein/protein interaction. Because the Flp-In™T-REx™-293 cells contain a single integrated FRT site, all of the hygromycin-resistant foci that we obtained after cotransfection with the pcDNA5/FRT/TO expression vector and pOG44 should be isogenic (i.e. the pcDNA5/FRT/TO expression vector should integrate into the same

genomic locus in every clone; therefore, all clones should be identical). So,we can simply use polyclonal selection of isogenic cell lines to generate the cell line what we need rapidly.

To test the dosage effect for DOX-regulated gene expression, different amount of DOX (showed as the Fig. 3) was added and incubated the cells for 24 h at 37 ℃ before harvesting. The protein WCL were made and Western blotting was performed. The concentration from 0.001 μg/ml to 5 μg/ml DOX, all showed the spata3 expression, but 0.001 μg/ml showed a weak expression and 1 to 5 μg/ml showed similar higher expression. The expression from 0.001 to 1 μg/ml was gradually increased. So the expression level of spata3 can be modulated easily, we can choose wide range DOX (0.1–5 μg/ml) of the concentration for higher gene expression and choose 0.001 μg/ml or less to obtain very low spata3 expression.

Androgen receptor (AR) may play important roles in the prostate cancer, the male development and spermatogenesis. Androgens are steroid hormones that are necessary for normal male phenotype expression, including the outward development of secondary sex characteristics as well as the initiation and maintenance of spermatogenesis. Many physiological actions of androgens are mediated by AR, a member of the nuclear receptor superfamily. AR functions as a ligand-dependent transcription factor, regulating expression of an array of target genes that are important in male pubertal development and fertility [2,11]. *Spata3* showed expression at the mature testis [1]. Bioinformatics analysis showed spata3 have protein/protein interaction domain (data didn't show). Therefore we can use this established cell line to explore the relationship between spata3 and AR, and its role not only prostate cancer but also the male development and spermatogenesis.

In a word, we have successfully established the inducible cell line with the stable expression of Flp-In™T-REx™-spata3 and it is very useful for spata3 function identification. This technique also is the very useful reference for other protein complex(s) purification and function identification.

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可诱导的稳定表达 Spata3 细胞系的建立及特征

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摘要 *spata3*是一个在睾丸中特异性表达的基因, 可能与精子发生或生精细胞凋亡相关。为了进一步研究 Spata3 的功能, 将 *spata3* 克隆入经修饰的 pcDNA5/FRT/TO 表达载体, 应用 Flp-In™T-REx™-293 细胞系作为宿主细胞, 成功地构建了可被四环素或 Doxycline 诱导的稳定表达 Flp-In™T-REx™-sptat3 的细胞系。该细胞系在 *spata3* 基因 的 3' 端有 2×FLAG tag 和 2×His tag, 在缺乏可利用的 spata3 或其抗体的情况下,也能够很容易地应用商品化的 FLAG 抗体检测到 spata3 全长蛋白的表达。这种可诱导的稳定表达 Flp-In™T-REx™-spata3 的细胞系的建立, 不仅有利于 spata3 的分析鉴定和功能研究, 而且对于其他蛋白质的分离纯化和功能研究也有很好的借鉴作用。

关键词 *spata3*; 稳定细胞系; 蛋白质表达; 诱导

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