The Silencing of Septin 1 via Synthetic siRNAs in Schizosaccharomyces pombe

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Abstract: Septins are evolutionary conserved GTP-binding proteins and form heterooligomers in cells to interact with cytoskeleton. The main roles of septins in cell are cytokines, membrane interactions, vesicle trafficking and microtubule and actin organization. Non-coding RNAs are key players of gene expression regulation. Small-interfering RNAs (siRNAs) belong to non-coding RNAs, are characterized by sequence complementarity to their target mRNAs. siRNAs silence their targets via mRNA degradation or inhibition of translation. In S. pombe siRNAs can also propagate heterochromatin to silence genes located in centromeric regions. In this study, the aim was understanding the results of extrinsically induced siRNA silencing in S. pombe by using synthetic siRNAs specific to Septin 1 gene. siRNAs were introduced to cells by lipid-based vesicles and the alterations in Septin 1 expressions were monitored with qPCR. Septin 1 expression was reduced dramatically via direct siRNA introduction to cells. The interactions between cell morphology and the reduced level of Septin 1 were observed after accomplishing the silencing of Septin 1 gene. In this study, it is shown that the morphology of the cells is affected by the reduced levels of Septin 1 expression and the cells form long and chain-like structures. This study constitutes an important example in understanding the results of inducing siRNA silencing extrinsically in S. pombe.

Keywords: RNAi, Fission Yeast, Septin, Cell Division

Introduction

Septins belong to guanine nucleotide-binding proteins that polymerize into filaments and have been recognized as important members of the cytoskeleton in recent years. They were first discovered in Saccharomyces cerevisiae through screening of gene mutations in cell-cycle progression, of which mutants caused morphological changes and disruption of cell division (Hartwell, 1971). Electron and fluorescence microscopy results revealed that septins localize in bud-neck separation as a ring and regulate cell division (Haarer and Pringle, 1987). Subsequently, septins were further characterized and were found conserved in all animals and fungi but not in plants (Kinoshita, 2003). Seven septin genes have been identified both in S. cerevisiae and Schizosaccharomyces pombe while there are 12 loci for septins in human genome (Kinoshita, 2003; Russell and Hall, 2011).

Inside the cell, septins form hetero-oligomeric filaments that are composed of repetitive tetrameric, hexameric and octameric structures (Spiliotis et al., 2008). They also generate rings and cage-like structures. These formations are functionally active in the cell and act as molecular scaffolds for recruiting other proteins and as diffusion barriers for cell compartmentalization (Mostowy and Cossart, 2012). Septins have been observed to associate with actin (Kinoshita et al., 2002), microtubules (Sellin et al., 2011) and phospholipid membranes (Bertin et al., 2010). These associations define the forms of high-order structures.

The main role of these assemblies is to act as subcellular scaffolds to mediate the accumulation of proteins involved during cytokinesis at the division site (Weems et al., 2014). They also play role in cell shape (Mostowy et al., 2011), cell polarity (Sirajuddin et al., 2007), cell movement (Gilden et al., 2012) and vesicle trafficking (Vega and Hsu, 2003).

The expression of septins is highly regulated in mammalian cells and they play critical roles in very diverse cellular functions, depending on their tissue-specific expressions and interacting molecules. Mutations and expression changes in septins have been associated with many cancer types and...
neurodegenerative diseases. Understanding the functional roles of septins is important for the diagnosis, prognosis and possible therapies for septin-related diseases (Peterson and Petty, 2010).

Yeasts have been used as model organisms in investigating cellular processes such as cell division, DNA replication, metabolism, protein folding and intracellular transport (Fields and Johnston, 2005). S. cerevisiae has been the pioneering yeast as the first eukaryotic organism of which the genome was sequenced (Goffeau et al., 1996). Among the other yeasts, especially S. pombe has started to contribute later to the understanding of the molecular basis of processes that are common in higher eukaryotes (Boilotin-Fukuhara et al., 2010). Even though S. cerevisiae has the leading role for genetic research, S. pombe has a feature that makes it more interesting. During evolution, S. pombe kept the RNA interference (RNAi) mechanism. Its genome encodes genes required for RNAi machinery and small non-coding RNAs, which regulate gene expression via post-transcriptional gene silencing and heterochromatin propagation (Reyes-Turcu and Grewal, 2012).

RNAi is an evolutionarily conserved mechanism for gene regulation in fungi, plants and animals. It uses non-coding, double-stranded RNAs for targeting mRNAs, depending on sequence homology and for silencing genes via degrading mRNAs or dispersing heterochromatin (Fire et al., 1998). Based on the biogenesis and functions of these small non-coding RNAs, they are classified mainly in four groups: Short interfering RNAs (siRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNAs) and long intervening non-coding RNAs, which regulate gene expression via post-transcriptional gene silencing and heterochromatin propagation (Reyes-Turcu and Grewal, 2012).

Materials and Methods

Strains and Growth Conditions

S. pombe972h- wild-type strains and leu2h+ mutant strains were grown as recommended by Gutz et al. (1974) in standard rich media (YEL) and Minimal (EMM) media containing additional leucine (50 mg L⁻¹) respectively.

Primers and siRNA Design for spn1 Gene

The sequence of spn1 gene was obtained from NCBI database and primers were selected based on the suggestions of NCBI Pick Primer software (Table 1). Three different siRNAs were designed by siRNA Wizard v3.1 software, provided by Invivogen. During siRNA design, siRNAs were selected from the 3′-UTR, 5′-UTR and exonic region of spn1 gene (Table 2). Selected siRNAs were produced commercially by Dharmacon ON-TARGET plus (Thermo Scientific). Before using they were dissolved in 5X siRNA Buffer (Thermo Scientific) as including 20 nmol siRNA in each solution.

siRNA Silencing of spn1 Gene

siRNA delivery to cells was performed by cationic liposomes using Dharma FECT siRNA Transfection Reagent (Thermo Scientific). First, siRNAs were diluted in 5X siRNA Buffer (Thermo Scientific) to 5 nmol and 50 μL of this solution was mixed with 750 μL YEL.
medium. Then, another 25 µL Dharma FECT siRNA Transfection Reagent without siRNA was mixed with 725 µL YEL medium. Both solutions were incubated 5 min at room temperature, then mixed and incubated on a shaker at room temperature for 20 min. After incubation, the mixture was added to 10 mL cell solution that includes 10⁶ cells/ml. The cells were incubated in a shaker at 30°C for 4 h for siRNA silencing.

**RNA Isolation and cDNA Synthesis**

Following the silencing, total RNA isolation was performed using Gene JET RNA Purification Kit, according to manufacturer’s instructions. Prior to using the kit, the cells were mechanically homogenized by vigorous shaking using glass beads and PBS.

**Gene Expression Analysis with Real-Time PCR**

The changes in mRNA levels of *spn1* gene following siRNA silencing were investigated by Real-Time PCR using *S. pombe* actin primers as a housekeeping gene (Table 3) and SYBR Green I for fluorescent marker. The reaction was performed in Light Cycler 480 instrument (Roche). The *spn1* primers and cDNAs from samples were added to Maxima SYBR Green qPCR Master Mix (Thermo Scientific), according to manufacturer’s instructions. Three biological and three technical replicas were performed for each samples.

Table 1. *spn1* primers for qPCR

| Primers | G+C content (%) | T<sub>m</sub> (°C) | Sequence (5′→3′) |
|---------|-----------------|------------------|------------------|
| Primer-F | 55              | 60.04            | GGGTTCGACGTTGATTCT |
| Primer-R | 50              | 59.97            | AAAATCGCCAAAACCGGTG |

Table 2. siRNAs for *spn1* silencing

| siRNA | Sequence (5′→3′) | Starting position | G+C content (%) |
|-------|------------------|------------------|-----------------|
| 1     | GCGTCAGTTGAACGGATACGT | 146              | 52.38           |
| 2     | ACCCACCGACCCAGAAATCTT | 301              | 52.38           |
| 3     | GAAGAGGAGCGCGTTCCTACAC | 1098             | 57.14           |

Table 3. *actin* primers for qPCR

| Primers | G+C Content (%) | T<sub>m</sub> (°C) | Sequence (5′→3′) |
|---------|-----------------|------------------|------------------|
| Primer-F | 50              | 56.1             | AGATTCTCATGGAGCGTGGT |
| Primer-R | 45              | 54.2             | TCAAAGTCCAAAGCGACGTA |

Fig. 1. pDQ105 plasmid for *S. pombe* transformation (ori: Origin of Replication, LEU2: Leucine 2 gene in yeast for selection, nmt1-p and nmt1-t: Thiamine promoter for yeast, GFPs65t: GFP gene, a2-tub: a2 tubulin gene)
Cell Growth Analysis after siRNA Silencing

Following the siRNA silencing described above, the growth of cells were measured spectrophotometrically. The optical density of cells was measured in every 2 h for 36 h at 600 nm wavelength. Three biological and three technical replicas were performed for each samples.

Plasmid Transformation to S. Pombe leu2h+ Cells

The pDQ105 plasmid was designed by Dr. Da-Qiao Ding and was acquired from National Bioresource Project, Yeast Genetic Resource Center Japan (YGRC/NBRP). The plasmid includes coding sequence of GFP-S65T adjacent to α2-tubulin and nmt1 promoter for the expression (Fig. 1). The transformant colonies were selected by the ampicillin resistance gene in E. coli and by leucine 2 gene in S. pombe leu2h+ cells. The plasmid was transformed to competent E. coli DH5α cells via heat-shock method and plasmids were isolated from E. coli colonies on LBA + ampicillin media using ZyppyTM Plasmid Miniprep Kit (Zymo Research). Then plasmids were transformed to S. pombe cells using Lithium Acetate Method (Moreno et al., 1991) and the colonies were grown on EMM + leucine media.

Fluorescent Microscopy Analysis of spn1 Silencing

Transformant S. pombe leu2h+ cells were subjected to siRNA silencing by siRNA 3 as described above and the effects of silencing on phenotype have been analysed using Olympus BX53F fluorescent microscope. The cells were collected in the middle of the logarithmic phase and fixed by alcohol, according to Forsburg and Rhind (2006) method. In order to acquire fluorescent signals from the cells the U-FBW filter was used at 489 nm excitation wavelength and 509 nm emission wavelength, in 30 ms exposure time.

Results

The Expression Levels of spn1 were Reduced by Synthetic siRNAs

Three siRNAs from the 5′-UTR, exonic region and 3′-UTR of spn1 gene were transferred to S. pombe 972h- cells separately, as combinations of two siRNAs and three of them together into the cells via cationic liposomes. Real-Time PCR analysis that compares relative spn1 mRNA levels to actin mRNA levels showed that siRNA 3 which is complementary to the 3′-UTR of spn1 reduced the expression of spn1 the most. In addition to that, siRNA 2 which is complementary to the exonic region of the gene also reduced the expression of spn1. The combinations of siRNAs that include siRNA 3 had more silencing effect than other combinations (Fig. 2).

Cell Growth was Stalled after siRNA Silencing

In order to observe the effects of siRNA silencing on cell growth, the cells that were subjected to siRNA silencing with different siRNAs were incubated and their optical density was measured on 600 nm every 2 h. Based on the measurements, siRNA 3 caused a very long delay before entering the logarithmic phase compared to the effects of other siRNAs and also the optic density could not reach to the same level as the control group, indicating that cells could not grow or divide properly. Application of siRNA 1, combinations of two siRNAs and three siRNAs together showed moderate delay compared to control cells, however siRNA 2 showed no significant effect neither on entering logarithmic phase nor on cell number (Fig. 3).

Fig. 2. qPCR results after siRNA silencing (control: Cells without siRNA applications, siRNA applications were numbered by used siRNA in each sample respectively, old: Cells from a culture that was incubated more than 36 h)
Fig. 3. Cell growth curve analysis after siRNA introduction (control: Cells without siRNA applications, siRNA applications were numbered by used siRNA in each sample respectively, old: Cells from a culture that was incubated more than 36 h)

Fig. 4. Morphological analysis for changes in cell shape after siRNA silencing (A: Cells subjected to siRNA silencing, B: Control cells without siRNA silencing, Arrows: elongated and chain-like cells)

Fig. 5. Morphological Changes Following siRNA Silencing (A, B, C, D: Different samples from cells subjected to siRNA silencing, Arrows: Elongated and chain-like cells)
siRNA Silencing Caused Morphological Changes in Cell Shape

Based on acquired data from Real-Time PCR and cell growth analysis results, siRNA 3 that is complementary to 3'-UTR of spn1 gene, was selected for further analysis of silencing on cell shape. The S. pombe leu2h+ cells were first transformed with pDQ105 plasmid and then siRNA 3 was transferred into cells. After incubation of 4 h with siRNA 3, the morphology of cells was changed to elongated, chain-like and multinucleated cells. The changes in the morphology were observed under fluorescent microscope (Fig. 4 and 5). Additionally, the number of morphologically elongated cells was counted under light microscope and the ratio of long cells was found 12% (Table 4).

Discussion

In this study, it was aimed to silence the expression of spn1 gene in S. pombe via synthetic siRNAs. Septins were first identified in S. cerevisiae and associated with cell division. They are guanine nucleotide-binding proteins and form filaments in the cell (Pham et al., 2014). Septin filaments have a central role in cytokinesis, vesicular transport, cilia formation, phagocytosis, exocytosis, cell polarity, membrane dynamics, chromosome alignment and segregation, apoptosis and response to DNA damage (Weems et al., 2014). The expression of septins in the cell is regulated strictly both in a temporal and spatial manner and the changes in the expression lead to development of cancer, neurodegenerative diseases and infections. Due to their very crucial part in cellular processes it is very important to understand the molecular aspects of septin structure, function and interactions (Petersen and Petty, 2010).

In S. pombe, seven septin genes were identified and in this study Septin1 was selected to be investigated due to its central role in heterooligomeric formation of septin complexes (An et al., 2004) and its homology with Septin 6 in classification of septins in all organisms (Weirich et al., 2008). Human septin 6 is particularly important in neurodegenerative diseases due to its involvement in neuron migration, formation of axons and dendrites and synaptic activity (Hu et al., 2012).

In recent years, siRNA-mediated gene silencing studies have been increasingly focusing in characterization of gene functions and RNAi-based methods have become a very important tool for developing new therapeutic strategies for cancer and neurodegenerative diseases. RNAi-mediated control of gene expression is a conserved process in almost all organisms. However, among the model organisms, S. cerevisiae does not have this feature while in S. pombe, the functional molecular such as Dicer, Ago and RdRP are coded as single copies in the genome and this makes S. pombe a more important model organism for RNAi studies (Dang et al., 2011).

RNAi-based gene silencing strategies are carried out in three ways: (i) by hairpin-structure RNA transcription from a transgene, (ii) by convergent transcription of RNA from genes of interest and (iii) by direct introduction of double-stranded RNAs into cells. The last method has been used very frequently in mammalian cells but it is not very common in yeast (Li et al., 2010). Especially in S. pombe, PCR-based mutations or knock-out technologies are much more common than RNAi-based technologies and in that sense, direct introduction of siRNAs to S. pombe cells is a novel approach for gene expression research in yeast.

Non-viral siRNA transfer systems have been widely used in recent years. Amphiphilic molecules and nanoparticles (Ghosn et al., 2010) are the most prominent technologies among the transfer systems. Even though carrying siRNAs into the cells with nanoparticles shows increased efficiency, amphiphilic lipid molecules are being more widely performed, based on their ease of handling, low cost and adaptability (Conde et al., 2015).

In this study, three siRNAs were designed and synthesized complementary to 5'-UTR, exonic region and 3'-UTR of spn1 gene. The siRNAs were introduced to cells via cationic liposomes and gene expression levels of spn1 gene were analysed via Real-Time PCR. The highest decrease in spn1 mRNA was observed in the cells that were introduced by the siRNA which is complementary to 3'-UTR of spn1 gene. The expression of spn1 gene was also reduced by the siRNA that is complementary to the exonic region. It has been shown that in fungi, specifically in Mucor circinelloides, siRNAs can be derived from exons of genes (Nicolas et al., 2010) but this feature has not been documented in S. pombe before.

In order to investigate the interaction of siRNAs with each other, the combination of two siRNAs and all three siRNAs together were introduced to cells. The results revealed that all samples that have the siRNA with 3'-UTR complementarity reduced the level of expression more which also shows the potential of this specific siRNA. Cell growth curve results of siRNA applications supported...
the findings from gene expression analysis and it is thought that the siRNA which was designed from the 3'-UTR of spn1 gene could be a potential gene silencing agent via direct introduction of double-stranded siRNAs triggering the RNAi pathway in vitro in S. pombe cells. Depending on these data, cells were observed under fluorescent microscope for morphological changes. Long, chain-like cells with multiple nuclei were found following the siRNA application, thus proving the success of the silencing. These findings are in accordance with the previously documented observations with septin mutants which revealed that cell morphology changes based on arrested cytokines is that causes the formation of longer, chain-like and polynucleated cells (Wu et al., 2010).

Conclusion

This study plays an important role as a preliminary work for direct siRNA introduction to S. pombe cells which has not been documented before. For further analysis, specific antibodies for Septin 1 can be applied in order to evaluate the protein expression of Septin 1 gene after siRNA introduction and other types of septins should also be silenced via siRNAs in order to understand their combined effects in cells. The findings of this study are considered to lead a more intense investigation on siRNA research in S. pombe and the prospective outcomes of similar research will help to develop more structured use of synthetic siRNAs for gene silencing in yeast.

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Author’s Contributions

Semih Ekimler: Participated in all experiments, review literature and write manuscript.
Merve Yilmazer: Participated in all experiments.
Ercan Arican: Designed the research plan, organized the study and contributed to result analysis.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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