The Effect of *EFG1* Gene Silencing on Down-Regulation of *SAP5* gene, by Use of RNAi Technology

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Abstract- Efg1 transcription factor is believed to be the main regulator of hyphal formation under many different conditions. In addition, it is responsible for positive regulation of the expression of several hyphal-specific genes. *SAP5,* which encodes secreted aspartic proteinase, is one of the mentioned genes and is crucial for pathogenicity properties. In the present work we have established the experimental conditions for the use of siRNA in the diploid yeast *Candida albicans* in order to knock-down the *EFG1* gene expression as well as the Efg1-dependent gene, *SAP5*. The 19-nucleotide siRNA was designed according to cDNA sequence of *EFG1* gene in *C. albicans* and modified-PEG/LiAc method was applied for yeast transfection. To quantify the level of both *EFG1* and *SAP5* gene expression, the cognate mRNAs were measured in *C. albicans* by quantitative real-time RT-PCR and data was consequently analyzed by use of REST® software. Images taken by fluorescent microscopy method indicated the effectiveness of transfection. According to REST® software data analysis, expression of *EFG1* gene decreased about 2.5-fold using 500 nM of siRNA. A 7-fold decrease in *EFG1* gene expression was observed when applying 1 µM of siRNA (P<0.05). Consequently, the expression of *SAP5* was significantly down-regulated both in yeast treated with 500 and 1000 nM of siRNA (P<0.05). In conclusion, post-transcriptional gene silencing (PTGS) is likely to be considered as a promising approach to discover new gene targets so as to design fungal-specific antifungal agents, and it is strongly possible that we are taking the right way to battle with *C. albicans*-associated infections.

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Introduction

It is strongly assumed that the ability of *Candida albicans* to transition from yeast to hyphae is essential for pathogenicity (1). A variety of transcription factor which are important for filamentation have been identified so far (2-4). True hyphae are formed under a range of environmental conditions which reflects the variety of the microenvironments that the yeast encounters in the host. For example, neutral pH, presence of serum, 5% CO_2, N-acetyl-D-glucosamine (GlcNAc) and growth in an embedded matrix or in a microaerophilic conditions (5) can induce true hyphae formation. In general, a combination of the serum and the temperature of 37°C are powerful and robust signals for germ tube formation from yeasts cells (5). Although links between transcriptional regulators of *C. albicans* morphogenesis and molecular mechanisms that end in hyphal growth are still ambiguous, it is well known that a series of transcription factors such as Efg1 are responsible for positive regulation of hyphal-specific gene expression (6,7). Efg1 transcription factor is thought to be the key regulator of hyphal formation under many conditions, including serum, CO_2, neutral pH and GlcNAc in liquid media and also on solid media (6,8,9). Expression of some hyphal-specific genes such

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as *SAP4-6*, *ALS3*, *HWP1* and *RBT4* depends on Efg1 (2,7) and the products of these genes will collectively help the yeast to increase the special properties of hyphae and to be more virulent. Nevertheless, none of these genes are lonely required for hyphal formation and maintenance (5).

C. albicans is able to produce a family of 10-member secreted aspartic proteases among which Sap4, Sap5 and Sap6 are expressed upon hyphal formation. Eight Sap proteins including Sap5 are crucial for pathogenicity properties such as adhesion, invasion or host cell damage (10). Moreover, degradation of tissue barriers during invasion and destruction of host defense molecules are other responsibilities of aspartic proteases in *C. albicans* cells (10,11). Even though, it seems as if the importance of Saps for tissue invasion and damage is relay on the infection model (11). Recently, it has been claimed that only *SAP5*, but none of the other tested *SAP* genes, is detectably activated in RHE infection model (11).

To suppress gene function at the post-transcriptional level, recently, methods of RNA-mediated gene silencing is used in several cell systems (12,13) as well as in fungi (14-16). RNAi and related gene silencing pathways are initiated by either production or introduction of small RNAs (≈20-30 nucleotides) which their sequence are complementary to a part of target mRNA. Generation of siRNA from dsRNAs is mediated by the RNase III-like enzyme of the ribonuclease Dicer family. RNA-induced silencing complexes (RISCs) are then formed by associating of the siRNAs with members of the Argonaute family of proteins. RISCs use the siRNAs as guides for the sequence-specific silencing of messenger RNAs that contain complementary sequences through induction of the degradation of the mRNAs or repressing their translation (17,18).

In the present work we have established the experimental conditions for the use of siRNA in the diploid yeast *C. albicans*. In addition, down-regulation of the *SAP5* gene was investigated as a consequence of silencing of *EFG1* gene expression.

Materials and Methods

Strains

In the present study, *C. albicans* wild-type strain ATCC14053 was applied. The strain was cultured on yeast extract/ pepton/ dextrose (YPD) medium plates, incubated at 37 ° C for 24h and was maintained at 4°C until use.

siRNA

The 19-nucleotide siRNA was designed based on cDNA sequence of *EFG1* gene of *C. albicans*. The antisense and sense sequences are 5'-FAM-ACAUUGAGCAAUUUGGUUC-3' and 5'-GAACCAAAUUGCUCAAUGU-3', respectively. Unrelated siRNA, which is a scramble sequence of antisense strand, having a sequence 5'-AUAUGCGCAACAUUGACA-3' was synthesized as a negative control. (Metabion, Germany). Antisense strand of siRNA was 5'-fluoro modified with FAM so as to trace the siRNA localization in yeast cells. Sense/antisense annealing was performed in annealing buffer (30 mM HEPES-KOH pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, and 50 mM NH_4Ac) according to the protocol for siRNA annealing (http://www.metabion.com/downloads/siRNAannealing.pdf) to generate siRNA duplex with symmetric 2-nt 3' overhang.

C. albicans yeast culture and transfection

C. albicans strain was grown on yeast nitrogen base (YNB) medium at an initial absorbance of 0.2 at 623 nm (OD_{623}≈ 0.2). The culture was incubated at 37 ° C with gentle shacking for 18-20 h. Afterward, when most *C. albicans* cells underwent exponential phase of their lifecycle, a final yeast concentration of 3×10^7 cells/ml (OD_{623}≈ 0.6) was prepared and used for transfection. Cells were then harvested by centrifugation and washed twice with cold sterile PBS. The final solution was divided into microtubes in such a way that each 1.5 ml microtube contained 1 ml of the final solution (3×10^7 cells in concentration). Transfection was performed using modified-PEG/LiAc method. Briefly, to prepare competent yeasts, cells were re-suspended in 500 µl of 100 mM LiCl, mixed gently and centrifuged at 5000 rpm for 2 min. Afterward 240 µl 50% PEG 3350 and 36 µl 1.0 M LiCl were added to the pellet. Eighteen and 9 µl of annealed siRNA were added to the solution in order to get the final concentration of 500 and 1000 nM (1 µM), respectively. DEPC-treated water was lastly added in order to get a final volume of 360 µl. A positive control (untransfected yeast cells) and a negative control, (yeast cells treated with 1 µM of unrelated siRNA) were also run along with the experimental. Then cells suffered a heat shock in a water bath at 42°C for 40 min followed by centrifugation at 6000-8000 rpm for 30 sec and re-suspension in 500 µl of YNB medium so as to allow the transfected cells to be recovered. Finally cultures were incubated at 37 ° C without shacking for 15 h. Transfected yeasts were then subjected to further studies.

siRNA entrance confirmation

To ensure that the entrance of siRNA was successful samples of exponentially growing siRNA-treated *C. albicans* cultures were washed twice with sterile PBS. To remove any unused siRNA, samples were then treated with RNase (Takara, Japan) and subjected to fluorescent microscopy. Samples of 1 µM siRNA-treated cells were mounted on slides and examined. Fluorescence images were obtained using 100 objective lenses on an Olympus fluorescent microscope with the appropriate fluorescence filters. Photomicroscopy was performed using a Hitachi 12dp camera. The locations of labeled siRNA were then defined.

RNA extraction and quantitative Real-Time RT-PCR assay

The expression of both *EFG1* and *SAP5* genes was measured by quantitative Real-time RT-PCR. Total RNA was extracted from both siRNA-treated (*EFG1*-specific and unrelated siRNA) and untreated cells using FastPure™ RNA kit (TaKaRa, Japan) according to manufacturer's protocol. RNA concentrations and purity were determined spectrophotometrically (eppendorf biophotometer, Germany). Equal amount of RNA (1 µg in 20 µl) were subjected to cDNA synthesis by use of PrimeScript RT reagent Kit (TaKaRa, Japan). *EFG1* and *SAP5* primers were designed on the bases of published sequence of the *EFG1* (NCBI, Accession: XM_709104.1), and *SAP5* (NCBI, Accession: XM_714054.1) genes in *C. albicans*. The β-actin gene (*ACT1*) was used as endogenous reference gene. The sequences of forward and reverse primers are mentioned in table 1. Standard curves for each gene were established with 4-serially diluted cDNA, which was obtained from cells grown to mid-logarithmic phase at 37° C, using specific primers under the appropriate PCR conditions. Real-time RT-PCR was performed with a StepOnePlus Real-Time PCR system (Applied Biosystems, USA) and SYBR® Premix Ex Taq™ II was used as a reagent specifically designed for intercalator-based real time PCR using SYBR Green I. All PCR reaction mixtures contained the following: 10 µl SYBR® Premix Ex Taq II (2X), 2 µl of first strand cDNA, 0.4 µM of each primers, 0.4 µl of ROX Reference Dye (50X) and dH_2O up to the final volume of 20 µl. The program for amplification was 95°C for 30 sec as initial denaturation step followed by the 40-cycled PCR step consisting of 95°C for 5 sec and 60 °C for 30 sec. Negative control (water as template) was included in each run. Expression of each investigated gene was normalized to the housekeeping gene *ACT1* and analyzed by applying REST© (2008 V2.0.7) software. The software uses the comparative C_t method ($\Delta\Delta C_t$) to analyze data. Expression of *EFG1* and *SAP5* genes from cells grown under siRNA-treatment condition was indicated as relative expression to that of gene from untreated yeast cells. Each experimental condition was performed in duplicate and each experiment was repeated twice on two different days for reproducibility.

Results

Transfection and siRNA entrance confirmation

To assess the potential of RNAi as a means of gene silencing in *C. albicans*, we benefited from the modified PEG/LiAc method for double-stranded RNA to be introduced into the cells. Florescent microscopy method was used at the first step of evaluating the efficiency of *C. albicans* transfection and more important, to trace the siRNA localization in yeasts. Yeast cells were harvested 15 h post transfection, washed, mounted and visualized. Apparently, only yeast cells with labeled-siRNA inside were luminous enough to be traced. Most of the cells were successfully transfected. Figure 1 indicates the location of siRNA within the yeast cells and demonstrated that transfection was performed properly.

Quality control and effect of siRNA on EFG1 gene expression

EFG1, *SAP5* and *ACT1* mRNA levels were monitored over a 15 h period of yeasts incubation with both unrelated and *EFG1*-spesific siRNAs. Positive control, untreated *C. albicans* cells, was included in each run of experiment.

Table 1. PCR primers for real-time RT-PCR analysis.

Gene	Primer name	Sequence (5'-3')	PCR product size (bp)	GeneBank
EFG1	Fefg140	TgCCAATAATgTgTCggTTg	140	XM_709104.1
	Refg140	CCCATCTCTTCTACCACgTgTC		
ACT1	Fact110	ACggTATTgTTTCCAACTgggACg	110	XM_717232.1
	Ract110	TggAgCTTCggTCAACAAAACTgg		
SAP5	Fsap140	ggTgATgTggACAAAAgAggA	140	XM_714054.1
	Rsap140	CCACAAgTCAgAAgACCCAgT		

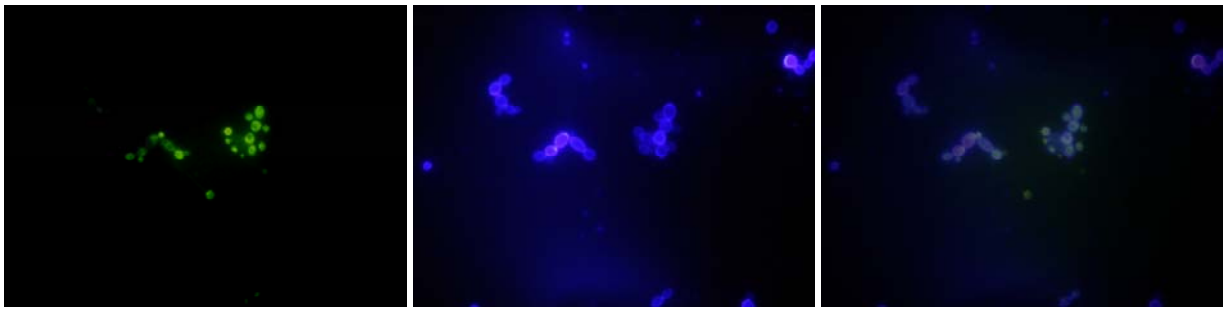

Figure 1. Fluorescent microscopy images of the cells transfected by siRNA specific to *EFG1* gene. From left to right: the fluorescent images of the yeasts carrying labeled-siRNA, calcofluor white staining of yeasts cell wall, and merged pictures of the two that indicates the location of siRNAs in the yeast cells.

EFG1, *SAP5* and *ACT1* primers demonstrated similar efficiency in titration experiment using *C. albicans* cDNA (1000 ng to 1000 pg) in serial dilutions (data not shown). Expression of each gene was indicated as expression ratio relative to that of untreated logarithmic-phase grown yeasts.

REST© (2008 V2.0.7) software was applied to analyze the obtained data of quantitative Real-time RT-PCR. On the basis of REST© output, down-regulation in the expression of *SAP5* gene were noteworthy in 1 µM siRNA-treated cells. There were significant differences between positive control (untreated cells) and test samples which were treated with 500 nM as well as with 1 µM of siRNA (P<0.05) (Figure 2). Expression of *EFG1* gene decreased about 2-fold using 500 nM of siRNA. A 5.5-fold decrease in *EFG1* gene expression was observed when applying 1 µM of siRNA (P<0.05).

Table 2. Output for relative expression of *EFG1* and *SAP5* genes by use of $\Delta\Delta C_t$ method (REST©, 2008 V2.0.7). Results indicate that the differences between *EFG1* expression in the yeast of control and sample groups are significant as well as the expression of *SAP5* gene.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
BetaAct	REF	1.0	1.000				
EFG1 (unrelated siRNA)	TRG	1.2	1.046	0.636 - 1.820	0.482 - 2.277	0.641	
EFG1 (500 nM siRNA)	TRG	1.2	0.357	0.209 - 0.674	0.153 - 0.858	0.000	DOWN
SAP5 (500 nM siRNA)	TRG	1.1	0.082	0.046 - 0.170	0.030 - 0.236	0.000	DOWN
EFG1 (1000 nM siRNA)	TRG	1.0	0.179	0.101 - 0.394	0.080 - 0.487	0.000	DOWN
SAP5 (1000 nM siRNA)	TRG	1.1	0.008	0.006 - 0.014	0.004 - 0.016	0.000	DOWN

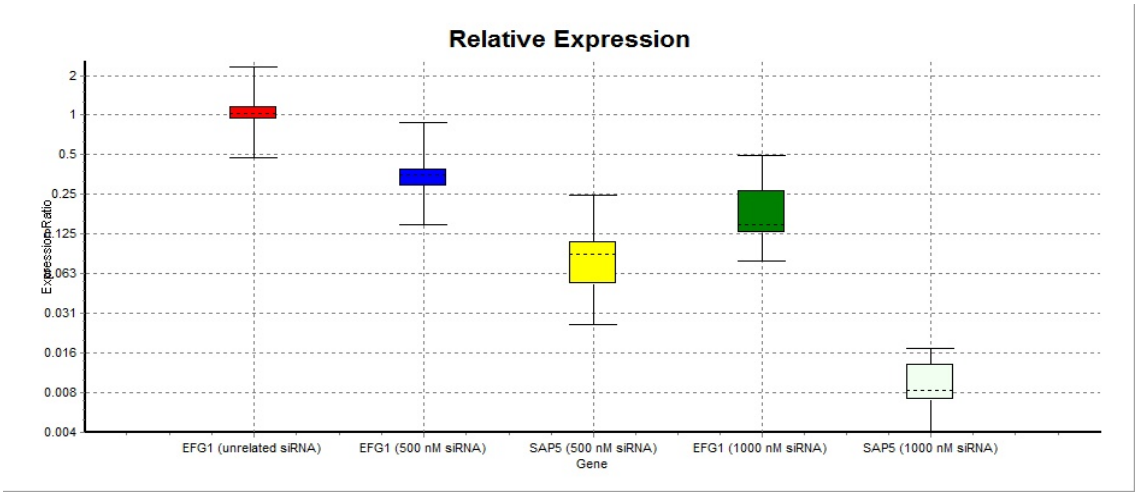

Figure 2. Effect of siRNA on *EFG1* and *ALS3* gene expression. (a) Unrelated siRNA, (b) 500nM treated siRNA, (c) 1 µM treated siRNA. Relative gene expression is indicates as expression ratio relative to that of untreated logarithmic-phase grown yeasts. Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

The level of *EFG1* mRNA in cells affected with unrelated siRNA was still detected as much as mRNA of positive control yeasts and seemed to be unchanged (P>0.05). Figure 2 indicates the relative expression ratio of *SAP5* and *EFG1* genes under different conditions. Table 2 shows the results of data analysis using REST© software.

Discussion

Among members of the genus *Candida*, *Candida albicans* is the most pathogenic. This species is equipped with several virulence factors such as genes coding adhesion molecules, metabolic flexibility, resistance to stressful conditions, yeast-to-hyphal transition, and production of hydrolytic and proteolytic enzymes (19). During invasion, up-regulation of some hyphal-associated genes including *ECE1*, *HWP1*, *SAP4-6*, *SOD5*, *ALS3*, *BMH2*, *FKH2*, and *SSN6* has been observed both during invasion *in vivo* infection of liver in mice and RHE models of infection (20). Some researchers found that proteases trigger invasion of *C. albicans* into human oral mucosa by degrading E-cadherin because E cadherin degradation was thoroughly inhibited in the presence of protease inhibitors (21). In this case, secreted aspartic proteases were implicated in tissue invasion (11). *SAP4-6* are considered as the genes up-regulated by a positive regulator of filamentous growth, Efg1 p (2). Accordingly, *efg1* mutant cells fail to express several *SAP* genes such as *SAP5* (22). It has been reported that only *SAP5*, but none of the other hyphal-specific *SAP* genes, was detectably activated in *in vitro* infection model using a recombination-based genetic reporter system (11). As Efg1 is a positive regulator of many other hyphal-specific genes which are essential for adhesion, invasion and cell damage, it is strongly possible that inhibition of *EFG1* gene expression may lead to attenuate the yeast pathogenicity through reducing both germ tube formation and destructive molecules expression. Therefore, in the present study we took advantages from RNAi technology to inhibit *EFG1* gene expression at the post transcriptional level.

Here, an *EFG1*-specific siRNA was designed and effectively presented to *C. albicans* cells by modified PEG/LiAc method and the level of *EFG1* and *SAP5* cognate mRNAs were measured by quantitative Real-time RT-PCR. Furthermore, the expression of *SAP5* as well as *EFG1* gene, have been investigated and significant reductions in both gene regulations were indicated. Analysis of qReal-time RT-PCR output demonstrated the outstanding influence of *EFG1* gene expression on pathogenicity of *C. albicans* yet again. Even a 2-fold decrease in *EFG1* gene expression can cause *SAP5* to be down-regulated.

Although some researches claimed that the RNAi pathway may be loss in the yeast *C. albicans* (23), our obtained results demonstrated that this pathway does exist functionally in *C. albicans*. It is a fact that due to the considerable variety in RNA silencing proteins among fungal species, RNA silencing pathways appear to have been diversified in this kingdom (24). In the case of *C. albicans*, according to CGD (Candida Genome Database), the genome of *C. albicans* hold a typical Argonaute homologue (orf19.2903; www.candidagenome.org), and a non-canonical Dicer (orf19.3796; www.candidagenome.org). Interestingly, the nucleotide sequence of the Dicer (orf19.3796) is only conserved among *Candida* species; however, the deduced amino acid sequence has quite high homology with ribonuclease of other fungal species. Moreover, the yeast *C. albicans* harbors a probable ribonuclease III protein. Remarkably, the homology of the amino acid sequences of ribonuclease III protein and the so called Dicer protein (orf19.3796) is 100% after performing alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The probable ribonuclease III protein contains DSRM and RIBOc domains. Despite that *Candida* Dicer lack a PAZ and helicase domain, it contains a Double-stranded RNA binding motif, DSRM and a Ribonuclease III C terminal domain, RIBOc. DSRM domain binding is not sequence specific but is highly specific for double stranded RNA. Considering above and out obtained results, it supposed that the Dicer of *C. albicans* is functional and effectively participate in RNAi silencing pathway. The Argonaute protein of the pathogenic yeast *C. albicans* holds both PiWi and PAZ domains which are essential motifs for Argonaut function and dsRNA binding. Therefore it is likely that the RNAi gene silencing pathway is functional in this microorganism. Nevertheless, it is highly recommended to apply this approach to knock-down other genes involved in viability or pathogenicity of *C. albicans*.

In conclusion, PTGS is likely to be considered as a promising approach to discover new gene targets so as to design fungal-specific antifungal agents, and it is strongly possible that we are taking the right way to battle with *C. albicans*-associated infections.

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References

1. Sudbery P, Gow N, Berman J. The distinct morphogenic states of Candida albicans. Trends Microbiol 2004;12(7):317-24.
2. Kumamoto CA, Vinces MD. Contributions of hyphae and hypha-co-regulated genes to Candida albicans virulence. Cell Microbiol 2005;7(11):1546-54.
3. Carlisle PL, Kadosh D. Candida albicans Ume6, a filament-specific transcriptional regulator, directs hyphal growth via a pathway involving Hgc1 cyclin-related protein. Eukaryot Cell 2010;9(9):1320-8.
4. Banerjee M, Thompson DS, Lazzell A, et al. UME6, a novel filament-specific regulator of Candida albicans hyphal extension and virulence. Mol Biol Cell 2008;19(4):1354-65.
5. Sudbery PE. Growth of Candida albicans hyphae. Nat Rev Microbiol 2011;9(10):737-48.
6. Stoldt VR, Sonneborn A, Leuker CE, et al. Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J 1997;16(8):1982-91.
7. Leng P, Lee PR, Wu H, et al. Efg1, a morphogenetic regulator in Candida albicans, is a sequence-specific DNA binding protein. J Bacteriol 2001;183(13):4090-3.
8. Lo HJ, Kohler JR, DiDomenico B, et al. Nonfilamentous C. albicans mutants are avirulent. Cell 1997;90(5):939-49.
9. Braun BR, Johnson AD. TUP1, CPH1 and EFG1 make independent contributions to filamentation in Candida albicans. Genetics 2000;155(1):57-67.
10. Naglik J, Albrecht A, Bader, et al. Candida albicans proteinases and host/pathogen interactions. Cell Microbiol 2004;6(10):915-26.
11. Lermann U, Morschhauser J. Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by Candida albicans. Microbiology 2008;154(Pt 11):3281-95.
12. Cottrell TR, Doering TL. Silence of the strands: RNA interference in eukaryotic pathogens. Trends Microbiol 2003;11(1):37-43.
13. Marques JT, Williams BRG. Activation of the mammalian immune system by siRNAs. Nat Biotechnol 2005;23(11):1399-405.
14. Janbon G, Maeng S, Yang DH, et al. Characterizing the role of RNA silencing components in Cryptococcus neoformans. Fungal Genet Biol 2010;47(12):1070-80.
15. Khatri M, Rajam MV. Targeting polyamines of Aspergillus nidulans by siRNA specific to fungal ornithine decarboxylase gene. Med Mycol 2007;45(3):211-20.
16. Rappleye CA, Engle JT, Goldman WE. RNA interference in Histoplasma capsulatum demonstrates a role for alpha-(1,3)-glucan in virulence. Mol Microbiol 2004;53(1):153-65.
17. Rao DD, Vorhies JS, Senzer N, et al. siRNA vs. shRNA: similarities and differences. Adv Drug Deliv Rev 2009;61(9):746-59.
18. Jinek M, Doudna JA. A three-dimensional view of the molecular machinery of RNA interference. Nature 2009;457(7228):405-12.
19. Calderone RA, Fonzi WA. Virulence factors of Candida albicans. Trends Microbiol 2001;9(7):327-35.
20. Zakikhany K, Thewes S, Wilson D, et al. From Attachment to Invasion: Infection Associated Genes of Candida albicans. Jpn J Med Mycol 2008;49(4):245-51.
21. Villar CC, Kashleva H, Nobile CJ, et al. Mucosal tissue invasion by Candida albicans is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. Infect Immun 2007;75(5):2126-35.
22. Korting HC, Hube B, Oberbauer S, et al. Reduced expression of the hyphal-independent Candida albicans proteinase genes SAP1 and SAP3 in the efg1 mutant is associated with attenuated virulence during infection of oral epithelium. J Med Microbiol 2003;52(Pt 8):623-32.
23. Staab JF, White TCMarr KA. Hairpin dsRNA does not trigger RNA interference in Candida albicans cells. Yeast 2011;28(1):1-8.
24. Nakayashiki H. RNA silencing in fungi: mechanisms and applications. FEBS Lett 2005;579(26):5950-7.