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# Gene expression by neuron restrictive silencer factor siRNA in KB human oral cancer cells

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구강암세포 KB에서 Neuron Restrictive Silencer Factor siRNA에 의한 유전자 발현

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조선대학교 대학원

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이 논문을 치의학 박사학위신청 논문으로 제출함.

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# 전우진의 박사학위논문을 인준함

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## 구강암세포 KB에서 Neuron Restrictive Silencer Factor siRNA에 의한 유전자 발현

**전 우 진**
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아연집게 단백질로 알려진 neuron restrictive silencer factor (NRSF)는 또한 RE-1 silencing transcription factor (REST)로 불리 우며, 비신경세포에서 neuron restrictive silencer element (NRSE)를 포함한 신경관련 유전자의 발현을 억제한다. 이는 비신경세포 뿐만 아니라 신경 전구세포에서도 발현 하지만 유사분열 후 신경에서는 발현되지 않는다. 그러므로 NRSF의 발현은 신경세포의 발달에 관련된 역할을 하며 신경관련 유전자의 발현을 유도함으로써 신경세포의 형성에 중요한 역할을 한다. 최근 들어 NRSF는 성숙된 신경세포에서 뿐만 아니라 암세포에서도 그 발현이 확인되었다. 그러나 구강암세포에서는 NRSE/NRSF의 조절 기전이 알려져 있지 않고 있다. 본 연구실에서는 선행연구로 NRSF가 구강암세포의 성장에 조절에 중요한 역할을 하고 있음을 확인하였고 NRSF의 발현을 siRNA를 이용하여 억제함으로써 구강암세포의 성장을 저해하고 있음을 확인하였다.

본 연구에서는 구강암세포 KB세포에서 NRSE/NRSF에 의한 조절 체계를 이해하기 위해 siRNA를 이용하여 KB세포의 NRSF의 발현을 억제한 후, microarray 분석을 통하여 유전자의 발현을 확인하였다. 다양한 유전자의 분석을 확인한 경과, 117개의 유전자가 그 발현이 증가하였고, 215개의 유전자가 발현이 억제되었다. 대부분의 발현이 증가된 유전자들은 signal transduction, cell communication, cell cycle, 그리고 apoptosis에 관여하는 유전자였으며 발현이 감소된 유전자들은 주로 RNA processing,

neurogenesis, transcription factor activity, 그리고 synaptogenesis에 관여하고 있음을 확인하였다. 흥미로운 사실은 NRSF가 전사조절 억제자로 알려져 있으나 구강암세포에서는 NRSF의 siRNA에 의해 200개 이상의 유전자가 그 발현이 감소하고 있었다. 또한 50% 이상의 유전자가 번역이나 ribosomal 단백질의 복합체를 이루는 유전자로 확인 할 수 있었다.

본 연구결과로 구강암세포에서 NRSF를 통한 새로운 성장 조절 기전을 확인할 수 있는 유전자들을 선별할 수 있었고 더 나아가, 이들 유전자의 조절 기전을 밝혀 새로운 기전을 확립 할 수 있을 것이다.

# I. Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of the oral and maxillofacial region, with more than 300,000 new cases reported annually worldwide. Based on currently available clinical assessment and treatment methods, patients are often diagnosed at a late stage of the disease and the 5-year survival rate has remained relatively low (50-0%) (Schliephake, 2003).

Surgical treatment for oral cancer can cause functional and aesthetic impairment, leading to withdrawal and social isolation (Hopper et al., 2004). Complications of radiotherapy can impair wound healing and further complicate surgical salvage after a failed procedure (Bodin et al., 2004). Conventional chemotherapeutic agents have been associated with numerous significant clinical complications, including nausea, hair loss, and pancytopenia; thus, alternative and less toxic chemical treatments for oral cancer are required (Yamachika et al., 2004).

The main risk factor for oral cancer is exposure to exogenous carcinogens such as tobacco smoke and alcohol. Annually, it is estimated that 127,459 deaths are caused from oral cavity cancer worldwide, of which 96,720 occur in developing countries (Ferlay et al., 2004). Oral squamous cell carcinoma (OSCC) is characterized by invasive and frequent perineural growth, a considerable rate of early recurrences, and frequent lymph nodes metastasis. Often these patients develop second primary cancers in the same or adjacent anatomical region.

Many patients are advanced-stage at diagnosis and incur significant morbidity and mortality due to the disease itself and the subsequent clinical management with its complications (Knauer, 2009;

Kowalski et al., 1997; Kowalski et al., 2005). Advances in cancer research have provided abundant newknowledge about cellular processes andmolecular biology in OSCC.

Our knowledge of carcinogenesis, identification of biological markers, and molecularlytargeted therapies is advancing through basic research, translational research and clinical trials, and ultimately analysis of factors specific to the individual andtheir tumormay result ineffective “personalized medicine” (Williams, 2010).

Since its discovery in 1995, the transcription repressor NRSF (otherwise called REST) has been investigated especially in relation to brain development (Ballas and Mandel 2005; Ooi and Wood 2007; Qureshi and Mehler 2009). NRSF is known to govern the expression ofhundreds of genes, many of which specific of nerve cells, including in their promoter (or other regulatory domain) a conserved, 21-5 bp sequence named NRSE. NRSF, also by its interaction with other transcription factors such as CREB (Wu and Xie 2006); Oct4, Sox2, and Nanog (Johnson et al. 2007); and various microRNAs (Wu and Xie 2006; Conaco et al. 2006; Yoo et al. 2009), orchestrates numerous epigenetic events in the course of neurogenesis (Ballas and Mandel 2005). Specifically, its rapid and large decrease during precursor maturation is instrumental to the establishment of the nerve cell phenotype. For quite some time, most studies on NRSF were focused on development, investigated by gene expression approaches and by the use as markers of single gene products. More recently, however, it has become clear that NRSF is not important only during nerve cell differentiation but remains critical also in mature nerve cells. Its increase in neurons during hypoxia has been interpreted as a mechanism contributing to cell death (Calderone et al. 2003; Formisano et al. 2003). Another increase, induced by the

glycolytic inhibitor 2-deoxy-D-glucose, was shown to play a neuron-protective effect in a model of epilepsy (Garriga-Canut et al. 2006).

Cell biologic studies were focused primarily on neurosecretion, investigated as a process typical of nerve cells. In pancreatic β cells, low NRSF was found to be needed for insulin secretion and the expression of other traits (Abderrahmani et al. 2004; Martin et al. 2008). The NRSF gene is expressed under the control of the Wnt system, working via the β-catening/TCF transcription system (Nihihara et al. 2002).

The protein, 116,00 in mol. wt, is rapidly turned over by proteolysis in the proteasome (half life *40 min, Westbrook et al. 2008). The different levels of NRSF in the various types of cells (from hardly appreciable in nerve cells to 50 fold higher in the others) tend to remain constant. In non-nerve cells, NRSF is expressed only in the canonical, full length form. In nerve cells, however, heterogeneity has been observed due to various types of alternative splicing. A truncate, noncanonical form which has attracted attention is NRSF4, very low in neurons (*1%: Palm et al. 1999) and higher in a fraction of neuroblastoma tumors. In order to function, NRSF needs to be translocated from the cytoplasm to the nucleus, a process dependent on its 11 aminoacid localization signal (Shimojo 2006). In spite of a few conflicting reports in the literature, the nuclear distribution of the repressor seems to be ubiquitous. In the nucleus, NRSF binds NRSE and induces the assembly of two effector complexes, localized at its N and C termini. NRSF4, bound to only the N terminal complex, probably works as a low affinity competitor of the full length factor (Magin et al. 2002; Tabuchi et al. 2002). The two effector complexes, necessary for NRSF action,

include cofactors (Sin3A at the N terminus, and CoREST at the C terminus) and the histone deacetylases 1 and 2. In addition, the most important C terminal complex includes methylating and de-methylating enzymes, some of which active on histones, others on the DNA (Ooi and Wood 2007).

Interestingly, when NRSF associates to different genes, and also to the same gene, the ratios of the various enzymes and regulatory proteins in the complexes can vary (Greenway et al. 2007; Klajn et al. 2009). Such a mechanism can account for the differential gene repressions by NRSF occurring in different types of cells, and in the same cells upon various treatments. In addition to the block of transcription, the epigenetic changes summarized so far can also induce structural consequences, such as a redistribution of nucleosomes followed by the transfer of the repressed genes from euchromatin to eterochromatin (Zheng et al. 2009).

Similar to the other transcription factors, NRSF works not only directly but also indirectly, through changes of expression/activity of one or more direct targets. Indirect effects can occur at the transcriptional level, mediated by the direct repression of other transcription factors. This mechanism most likely explains a few stimulatory effects induced by NRSF, for example, the increase of the corticotropin-releasing hormone (Seth and Majzoub 2001) and the increase of the enlargeosomes (Schulte et al. 2010), both observed in PC12 cells. Indirect effects can also take place at a posttranscriptional, cell-specific level. For example, in PC12 cells, the expression of the opioid l receptor was repressed by NRSF, whereas in the neuroblastoma NMB cells, it was increased via an indirect, post-transcriptional mechanism that in PC12 was apparently lacking (Kim et al. 2008).

In this study, to understand mechanism of cell growth regulation by NRSF expression, microarray analysis and gene screening was performed using NRSF siRNA in KB human oral cancer cells. In addition, as further study, quantitative PCR should be need to verify the microarray data.

# Ⅱ. Materials and Methods

## Ⅱ-1. Cell culture

Human oral squamous cell carcinoma cell line KB cells were grown in MEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin/100 μg/ml streptomycin (Gibco) and 0.1 mM MEM Non-Essential Amino Acids (Gibco) at 37 °C in a humidified atmosphere of 5% CO2.

## Ⅱ-2. siRNA and transfection

KB cells were seeded 24 h prior to transfection in 6-well plates at a density of 7×105 cells in each well. After overnight incubation, cells were transfected with 1 ㎍ of NRSF/REST siRNA using lipofectamine 2000 transfection reagent (invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, NRSF siRNA and lipofectamine 2000 were each diluted in 50 ㎕ Opti-MEM I and incubated for 5 min at room temperature. The diluted NRSF/REST siRNA and lipofectamine 2000 were combined and then incubated for 20 min at room temperature. After incubation, NRSF/REST siRNA/lipofectamine 2000 complexes were added to each well. The cells were incubated at 37℃ in a humidified atmosphere of 5% CO2. NRSF/REST siRNA and Negative control siRNA were purchased from Bioneer (AccuTarget™ Human genome-wide Predesigned siRNA; No. 1128323, AccuTarget™ Negative control siRNA; SN-1002, Daejeon, Korea). The concentrations of siRNAs were optimized to 50 pmol per well. After transfection, On days, cells were harvested and extracted total RNA for microarray analysis.

## Ⅱ-3. RNA preparation

Total RNA was prepared from cultured cells using the RNeasy total RNA purification system (Qiagen, MD, USA),its concentration quantified spectrophotometrically at 260 nm,and stored at )80 C.

## Ⅱ-4. DNA microarray analysis

For control and test RNAs, the synthesis of target cRNA probes and hybridization were performed using Agilent' s Low RNA Input Linear Amplification Kit PLUS (Agilent Technology) according to the manufacturer' s instructions. Briefly, each 0.5 ug total RNA was mixed with the diluted Spike mix and T7 promoter primer mix and incubated at 65° C for 10 min. cDNA master mix (5X First strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and MMLV-RT) was prepared and added to the reaction mixer. The samples were incubated at 40° C for 2 h and then the RT and dsDNA synthesis was terminated by incubating at 65° C for 15 min. The transcription master mix was prepared as the manufacturer' s protocol (4X Transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-Out, inorganic pyrophosphatase, T7-RNA polymerase, and Cyanine 3/5-CTP). Transcription of dsDNA was performed by adding the transcription master mix to the dsDNA reaction samples and incubating at 40° C for 2 h. Amplified and labeled cRNA was purified on RNase Mini Spin Columns (Qiagen, Hilden, Germany) according to the manufacturer' s protocol. Labeled cRNA target was quantified using ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). After checking labeling efficiency, each 750 ng of cyanine 3-labeled and cyanine

5-labeled cRNA target were mixed and the fragmentation of cRNA was performed by adding 10X blocking agent and 25X fragmentation buffer and incubating at 60° C for 30 min. The fragmented cRNA was resuspended with 2X hybridization buffer and directly pipetted onto assembled Agilent Whole Human Genome Oligo Microarray. The arrays hybridized at 65° C for 17 h using Agilent Hybridization Oven. The hybridized microarrays were washed as the manufacturer' s washing protocol (Agilent Technology).

## II-5. Microarray data analysis

The hybridization images were analyzed by Agilent DNA microarray Scanner and the data quantification was performed using Agilent Feature Extraction software. The average fluorescence intensity for each spot was calculated and local background was subtracted. All data normalization and selection of fold-changed genes were performed using GeneSpring GX 7.3 (Agilent Technology). Genes were filtered with removing flag-out genes in each experiment. Intensity-dependent normalization (LOWESS) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity vs. ratio curve. The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity.

# Ⅲ. Results

## Ⅲ-1. Gene expression profile in NRSF-siRNA treated KB cells

Previous study in our lab, NRSF is important factor to regulate the cell proliferation of KB human oral cancer cells. Cell growth was significantly inhibited by repression of NRSF expression using the NRSF specific target siRNA treatment in KB cells. Therefore, to understand the NRSE/NRSF regulatory network in KB cells, we performed the microarray analysis by NRSF specific targeted siRNA treatment in KB human oral cancer cells.

To screen the genes of NRSF siRNA-mediated cell regulation, global gene expression profiling was performed at 24 h post-transfection, when NRSF mRNA was most effectively suppressed (data not shown). Results were clustered, based on the differential expression level (2-fold up or down), and visualized using a color scale (Figure 1). Microarray software analysis showed that upregulated genes were identified by 117 probe sets, whereas 215 genes were downregulated at least 2-fold, compared with the control (Table.1 and 2).

As shown in table 1 and 2, although NRSF has been known as a repressor to silence neuronal gene in nonneurol cells, our data showed numbers of downregulated genes were much more than upregulated genes by NRSF siRNA treatment in KB cells. And also, NRSF protein in KB cells may be involved in a lot of pathway as well as transcriptional regulation. More than 50 % of NRSF controled genes in both upregulaed and downregulated

genes were included in signal transduction (21 genes of upregulation genes), protein metabolic process (102 genes in downregulated genes) and translational pathway (57 genes in downregulated genes).

Table 1. Upregulated gene categories by NRSF siRNA treated KB cells.

| Gene Category | Count |
|---|---|
| Regulation of signal transduction | 21 |
| Regulation of defense response | 11 |
| Positive regulation of cell communication | 10 |
| Secretion by cell | 9 |
| Exocytosis | 8 |
| Positive regulation of defense response | 7 |
| Positive regulation of immune response | 6 |
| Regulation of innate immune response | 5 |
| Positive regulation of response to external stimulus | 5 |
| Regulation of inflammatory response | 5 |
| Regulation of vesicle-mediated transport | 5 |
| Regulation of blood pressure | 5 |
| Positive regulation of inflammatory response | 4 |
| Response to hydrogen peroxide | 4 |
| Positive regulation of cell cycle | 4 |
| Regulation of neurotransmitter levels | 4 |
| Male meiosis | 3 |
| Regulation of systemic arterial blood pressure mediated by a chemical signal | 3 |
| Apoptotic mitochondrial changes | 3 |
| Angiotensin catabolic process in blood | 2 |
| Multicellular organismal protein catabolic process | 2 |
| Multicellular organismal protein metabolic process | 2 |
| Regulation of angiotensin metabolic process | 2 |
| Positive regulation by symbiont of host defense response | 2 |
| Modulation by organism of defense response of other organism during symbiotic interaction | 2 |
| Modulation by symbiont of host defense response | 2 |
| Positive regulation by organism of defense response of other organism during symbiotic interaction | 2 |

Table 2. Downregulated gene categories by NRSF siRNA treated KB cells.

| Gene Category | Count |
|---|---|
| Cellular protein metabolic process | 102 |
| Translation | 57 |
| Translational elongation | 50 |
| RNA metabolic process | 32 |
| Positive regulation of cellular biosynthetic process | 25 |
| Positive regulation of biosynthetic process | 25 |
| RNA processing | 24 |
| Tissue development | 23 |
| Neurogenesis | 21 |
| Positive regulation of cell death | 19 |
| Positive regulation of apoptosis | 18 |
| Positive regulation of programmed cell death | 18 |
| Negative regulation of apoptosis | 14 |
| Negative regulation of programmed cell death | 14 |
| Negative regulation of cell death | 14 |
| Anti-apoptosis | 10 |
| Positive regulation of hydrolase activity | 9 |
| Response to insulin stimulus | 8 |
| Regulation of transcription factor activity | 7 |
| Regulation of cellular catabolic process | 6 |
| Positive regulation of DNA binding | 6 |
| Positive regulation of cytokine production | 6 |
| Regulation of skeletal muscle fiber development | 5 |
| Regulation of skeletal muscle tissue development | 5 |
| Regulation of striated muscle cell differentiation | 5 |
| Regulation of muscle cell differentiation | 5 |
| Regulation of striated muscle tissue development | 5 |
| Positive regulation of cell cycle | 5 |
| Post-Golgi vesicle-mediated transport | 5 |
| Skeletal muscle organ development | 5 |
| Positive regulation of protein transport | 5 |
| Cellular response to insulin stimulus | 5 |
| Hexose catabolic process | 5 |
| Regulation of myeloid cell differentiation | 5 |
| Monosaccharide catabolic process | 5 |

| | |
|---|---|
| Long-term strengthening of neuromuscular junction | 4 |
| Regulation of synaptic growth at neuromuscular junction | 4 |
| Regulation of synaptogenesis | 4 |
| Regulation of synapse structure and activity | 4 |
| Regulation of cytokine secretion | 4 |
| Positive regulation of synaptic transmission | 4 |
| Positive regulation of cellular catabolic process | 4 |
| Insulin receptor signaling pathway | 4 |
| Positive regulation of transmission of nerve impulse | 4 |
| Positive regulation of neurological system process | 4 |
| Positive regulation of protein secretion | 4 |
| Regulation of myeloid leukocyte differentiation | 4 |
| Osteoblast differentiation | 4 |
| Translational initiation | 4 |
| Striated muscle contraction | 4 |
| Negative regulation of RNA splicing | 3 |
| Ribosome assembly | 3 |
| Regulation of interleukin-12 production | 3 |
| Negative regulation of myeloid leukocyte differentiation | 3 |
| Myoblast differentiation | 3 |
| Centrosome cycle | 3 |
| Positive regulation of glucose metabolic process | 3 |
| Positive regulation of carbohydrate metabolic process | 3 |
| Regulation of RNA splicing | 3 |
| Positive regulation of cellular carbohydrate metabolic process | 3 |
| Regulation of osteoclast differentiation | 3 |
| Thymus development | 3 |
| Ribosomal small subunit export from nucleus | 2 |
| Ribosome export from nucleus | 2 |
| Establishment of ribosome localization | 2 |
| Ribosomal subunit assembly | 2 |

Two-dimensional scatter plot analysis of gene expression values for all genes on the NRSF siRNA-transfected cells and control cells from the microarray. Red dots represent upregulated genes and green dots represent downlagulated gene at least 2-fold by NRSF siRNA treatment compared with the control. In addition, three NRSF siRNA treated samples of scatter plots showed similar patterns. We then proceeded to classify potential

NRSF target genes indentified with functional category. As shown in table 2, even though, NRSF is functions as a transcriptional repressor, downregulated genes were more identified in NRSF siRNA treated cells. NRSF target genes were involved in a lot of different categories pathway as cell cycle, inflammatory, signal transduction, apoptosis and RNA metabolic etc.

Interestingly, although NRSF has been known as a transcriptional repressor to silence neuronal gene, out data shown that more than 200 genes were downregulated by NRSF siRNA treated KB cells. Specially, 50 % of these genes are involved in translational regulation and consist of ribosomal complex proteins (Fig. 3)

### III-2. Gene expression data of microarray analysis by NRSF siRNA treated KB human oral cancer cells.

Previous our study showed that NRSF siRNA treatment led to inhibit the cell proliferation and translation. In this data, we could not found cell groth and translational related genes in upregulated genes by NRSF siRNA treated KB cells (Table 3). In contrast, as shown in Table 4, A lot of translational related genes, specially ribosomal genes were downregulated by NRSF siRNA treatment in KB cells. This data indicated that NRSF can mainly control the translational pathway in KB cells.

Table 3. Upregulated genes of microarray analysis by NRSF siRNA

| GENE SYMBOL | GENE NAME | FOLD |
|---|---|---|
| BSN | bassoon (presynaptic cytomatrix protein) | 9.46 |
| AP3B2 | adaptor-related protein complex 3, beta 2 subunit | 9.36 |
| IFI44L | interferon-induced protein 44-like | 7.75 |
| MX1 | myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) | 7.19 |
| IFI27 | interferon, alpha-inducible protein 27 | 6.78 |
| UNC13A | unc-13 homolog A (C. elegans) | 4.74 |
| IFI27 | interferon, alpha-inducible protein 27 | 6.62 |
| UNC13A | unc-13 homolog A (C. elegans) | 4.85 |
| IFI6 | interferon, alpha-inducible protein 6 | 6.48 |
| SCAMP5 | secretory carrier membrane protein 5 | 4.93 |
| CPLX1 | complexin 1 | 5.06 |
| OAS2 | 2'-5'-oligoadenylate synthetase 2, 69/71kDa | 4.90 |
| TMEM198 | transmembrane protein 198 | 4.28 |
| OAS2 | 2'-5'-oligoadenylate synthetase 2, 69/71kDa | 3.81 |
| ISG15 | ISG15 ubiquitin-like modifier | 4.10 |
| SAMD9L | sterile alpha motif domain containing 9-like | 3.82 |
| STMN3 | stathmin-like 3 | 3.33 |
| DISP2 | dispatched homolog 2 (Drosophila) | 3.16 |
| IFIT5 | interferon-induced protein with tetratricopeptide repeats 5 | 3.51 |
| TEP1 | telomerase-associated protein 1 | 2.93 |
| IFI44 | interferon-induced protein 44 | 3.05 |
| MLLT1 | myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 1 | 5.53 |
| TMEM145 | transmembrane protein 145 | 3.60 |
| HERC6 | hect domain and RLD 6 | 3.10 |
| C19orf30 | chromosome 19 open reading frame 30 | 4.72 |
| SNAP25 | synaptosomal-associated protein, 25kDa | 2.78 |
| IFITM1 | interferon induced transmembrane protein 1 (9-27) | 2.70 |
| RPRML | reprimo-like | 4.35 |
| GNRHR | gonadotropin-releasing hormone receptor | 1.09 |
| CCDC123 | coiled-coil domain containing 123 | 4.19 |
| DSTYK | dual serine/threonine and tyrosine protein kinase | 5.67 |
| GNG4 | guanine nucleotide binding protein (G protein), gamma 4 | 2.52 |
| TAC3 | tachykinin 3 | 3.09 |
| IFIT5 | interferon-induced protein with tetratricopeptide repeats 5 | 2.42 |
| GDAP1 | ganglioside-induced differentiation-associated protein 1 | 3.73 |

| | | |
|---|---|---|
| CCL5 | chemokine (C-C motif) ligand 5 | 2.48 |
| KCNK3 | potassium channel, subfamily K, member 3 | 2.59 |
| KLHL18 | kelch-like 18 (Drosophila) | 2.99 |
| SSTR4 | somatostatin receptor 4 | 2.31 |
| SPRR2D | small proline-rich protein 2D | 1.87 |
| STAT1 | signal transducer and activator of transcription 1, 91kDa | 2.55 |
| SAMD9 | sterile alpha motif domain containing 9 | 3.44 |
| C9orf109 | chromosome 9 open reading frame 109 | 2.88 |
| NKAIN1 | Na+/K+ transporting ATPase interacting 1 | 2.28 |
| FAM123A | family with sequence similarity 123A | 2.71 |
| IFIH1 | interferon induced with helicase C domain 1 | 2.13 |
| C4orf12 | chromosome 4 open reading frame 12 | 3.72 |
| NCR3 | natural cytotoxicity triggering receptor 3 | 2.34 |
| PSMD10 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 10 | 2.08 |
| STMN3 | stathmin-like 3 | 2.11 |
| DDX60 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 | 1.85 |
| TMEM151A | transmembrane protein 151A | 2.64 |
| FAM40B | family with sequence similarity 40, member B | 2.05 |
| C8orf84 | chromosome 8 open reading frame 84 | 2.62 |
| BATF2 | basic leucine zipper transcription factor, ATF-like 2 | 1.89 |
| SUB1 | SUB1 homolog (S. cerevisiae) | 3.04 |
| STMN3 | stathmin-like 3 | 1.96 |
| ADARB1 | adenosine deaminase, RNA-specific, B1 (RED1 homolog rat) | 1.37 |
| S100A7 | S100 calcium binding protein A7 | 2.21 |
| ODF4 | outer dense fiber of sperm tails 4 | 2.55 |
| ATF7IP2 | activating transcription factor 7 interacting protein 2 | 3.04 |
| MYO1A | myosin IA | 1.93 |
| RHO | rhodopsin | 2.50 |
| C5orf58 | chromosome 5 open reading frame 58 | 2.92 |
| C14orf184 | chromosome 14 open reading frame 184 | 1.18 |
| DDX60 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 | 1.83 |
| IL23R | interleukin 23 receptor | 3.18 |
| ELAVL4 | ELAV (embryonic lethal, abnormal vision, Drosophila)-like 4 (Hu antigen D) | 5.86 |
| IRF9 | interferon regulatory factor 9 | 2.12 |
| OAS3 | 2'-5'-oligoadenylate synthetase 3, 100kDa | 2.13 |
| IFIT2 | interferon-induced protein with tetratricopeptide repeats 2 | 1.98 |
| HERC6 | hect domain and RLD 6 | 2.39 |

| | | |
|---|---|---|
| LOC100130729 | hypothetical protein LOC100130729 | 0.82 |
| SLC15A3 | solute carrier family 15, member 3 | 2.18 |
| GBP1 | guanylate binding protein 1, interferon-inducible, 67kDa | 2.28 |
| IRF7 | interferon regulatory factor 7 | 1.68 |
| HCN3 | hyperpolarization activated cyclic nucleotide-gated potassium channel 3 | 1.98 |
| IFIT1 | interferon-induced protein with tetratricopeptide repeats 1 | 1.92 |
| KCNK3 | potassium channel, subfamily K, member 3 | 2.12 |
| DMC1 | DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast) | 3.64 |
| TFR2 | transferrin receptor 2 | 1.94 |
| ATL1 | atlastin GTPase 1 | 1.91 |
| DDX60L | DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like | 2.00 |
| FLJ30064 | hypothetical protein LOC644975 | 2.43 |
| ZCWPW1 | zinc finger, CW type with PWWP domain 1 | 1.77 |
| TTTY14 | testis-specific transcript, Y-linked 14 (non-protein coding) | 2.21 |
| ARHGEF15 | Rho guanine nucleotide exchange factor (GEF) 15 | 2.12 |
| C9orf110 | chromosome 9 open reading frame 110 | 2.16 |
| SLC22A23 | solute carrier family 22, member 23 | 2.58 |
| CAPZA1 | capping protein (actin filament) muscle Z-line, alpha 1 | 1.90 |
| APOL3 | apolipoprotein L, 3 | 2.40 |

Table 4. Downregulated genes of microarray analysis by NRSF siRNA

| GENE SYMBOL | GENE NAME | FOLD |
|---|---|---|
| TMEM178 | transmembrane protein 178 | 0.49 |
| H2AFZ | H2A histone family, member Z | 0.49 |
| CA12 | carbonic anhydrase XII | 0.49 |
| KAT2B | K(lysine) acetyltransferase 2B | 0.49 |
| MYL6 | myosin, light chain 6, alkali, smooth muscle and non-muscle | 0.49 |
| EIF1 | eukaryotic translation initiation factor 1 | 0.49 |
| TOMM7 | translocase of outer mitochondrial membrane 7 homolog (yeast) | 0.49 |
| CUTA | cutA divalent cation tolerance homolog (E. coli) | 0.49 |
| NDUFA4 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa | 0.49 |
| S100P | S100 calcium binding protein P | 0.49 |
| UBC | ubiquitin C | 0.49 |
| CUEDC1 | CUE domain containing 1 | 0.49 |
| RPL35 | ribosomal protein L35 | 0.49 |
| NPM1 | nucleophosmin (nucleolar phosphoprotein B23, numatrin) | 0.49 |
| TAOK1 | TAO kinase 1 | 0.48 |
| HIST1H3D | histone cluster 1, H3d | 0.48 |
| RBM19 | RNA binding motif protein 19 | 0.48 |
| GTPBP6 | GTP binding protein 6 (putative) | 0.48 |
| MYO1C | myosin IC | 0.48 |
| PABPC1 | poly(A) binding protein, cytoplasmic 1 | 0.48 |
| SECTM1 | secreted and transmembrane 1 | 0.48 |
| HOMER1 | homer homolog 1 (Drosophila) | 0.48 |
| PIK3CB | phosphoinositide-3-kinase, catalytic, beta polypeptide | 0.48 |
| LY6K | lymphocyte antigen 6 complex, locus K | 0.48 |
| PPIAL4A | peptidylprolyl isomerase A (cyclophilin A)-like 4A | 0.47 |
| CRYBG3 | beta-gamma crystallin domain containing 3 | 0.47 |
| RPL4 | ribosomal protein L4 | 0.47 |
| CCT4 | chaperonin containing TCP1, subunit 4 (delta) | 0.47 |
| HSPB1 | heat shock 27kDa protein 1 | 0.47 |
| RPS27A | ribosomal protein S27a | 0.47 |
| RPS7 | ribosomal protein S7 | 0.47 |
| FAM43A | family with sequence similarity 43, member A | 0.47 |
| C21orf67 | chromosome 21 open reading frame 67 | 0.46 |
| RPLP0 | ribosomal protein, large, P0 | 0.46 |
| HSPD1 | heat shock 60kDa protein 1 (chaperonin) | 0.46 |

| | | |
|---|---|---|
| RPL10 | ribosomal protein L10 | 0.46 |
| KRT18 | keratin 18 | 0.46 |
| CHIC1 | cysteine-rich hydrophobic domain 1 | 0.46 |
| IGF1 | insulin-like growth factor 1 (somatomedin C) | 0.46 |
| MED13L | mediator complex subunit 13-like | 0.46 |
| GNAS | GNAS complex locus | 0.46 |
| RPSA | ribosomal protein SA | 0.46 |
| KBTBD10 | kelch repeat and BTB (POZ) domain containing 10 | 0.46 |
| COX1 | cytochrome c oxidase subunit I | 0.46 |
| RPL23A | ribosomal protein L23a | 0.45 |
| RPL30 | ribosomal protein L30 | 0.45 |
| TRDMT1 | tRNA aspartic acid methyltransferase 1 | 0.45 |
| RPL18A | ribosomal protein L18a | 0.45 |
| PRUNE | prune homolog (Drosophila) | 0.45 |
| RPS13 | ribosomal protein S13 | 0.45 |
| STK39 | serine threonine kinase 39 (STE20/SPS1 homolog, yeast) | 0.45 |
| RPS29 | ribosomal protein S29 | 0.45 |
| VIM | vimentin | 0.45 |
| COX6B1 | cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous) | 0.45 |
| C18orf1 | chromosome 18 open reading frame 1 | 0.44 |
| RPS2P32 | ribosomal protein S2 pseudogene 32 | 0.44 |
| RPL8 | ribosomal protein L8 | 0.44 |
| RAB17 | RAB17, member RAS oncogene family | 0.44 |
| RPL14 | ribosomal protein L14 | 0.44 |
| NUDT16L1 | nudix (nucleoside diphosphate linked moiety X)-type motif 16-like 1 | 0.44 |
| RPL39 | ribosomal protein L39 | 0.44 |
| UNC5B | unc-5 homolog B (C. elegans) | 0.44 |
| CYTB | cytochrome b | 0.44 |
| RPS16 | ribosomal protein S16 | 0.44 |
| FAU | Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed | 0.44 |
| RPS20 | ribosomal protein S20 | 0.44 |
| UBB | ubiquitin B | 0.44 |
| UBA52 | ubiquitin A-52 residue ribosomal protein fusion product 1 | 0.44 |
| RPL24 | ribosomal protein L24 | 0.43 |
| PTPN21 | protein tyrosine phosphatase, non-receptor type 21 | 0.43 |
| RPS10 | ribosomal protein S10 | 0.43 |
| RPS2 | ribosomal protein S2 | 0.43 |
| RPL18A | ribosomal protein L18a | 0.43 |
| RPL23A | ribosomal protein L23a | 0.43 |

| | | |
|---|---|---|
| RPS25 | ribosomal protein S25 | 0.43 |
| TMEM209 | transmembrane protein 209 | 0.43 |
| RPL9 | ribosomal protein L9 | 0.43 |
| ATP5G2 | ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9) | 0.43 |
| DCBLD2 | discoidin, CUB and LCCL domain containing 2 | 0.43 |
| CRB1 | crumbs homolog 1 (Drosophila) | 0.43 |
| RCOR2 | REST corepressor 2 | 0.43 |
| RPL36AL | ribosomal protein L36a-like | 0.43 |
| NACA | nascent polypeptide-associated complex alpha subunit | 0.43 |
| RPS25 | ribosomal protein S25 | 0.43 |
| RPSA | ribosomal protein SA | 0.43 |
| RPL3 | ribosomal protein L3 | 0.42 |
| RPL23AP82 | ribosomal protein L23a pseudogene 82 | 0.42 |
| STK39 | serine threonine kinase 39 (STE20/SPS1 homolog, yeast) | 0.42 |
| RPL14 | ribosomal protein L14 | 0.42 |
| RPSA | ribosomal protein SA | 0.42 |
| RPS15A | ribosomal protein S15a | 0.42 |
| CYBRD1 | cytochrome b reductase 1 | 0.42 |
| NACA | nascent polypeptide-associated complex alpha subunit | 0.42 |
| ATP1B3 | ATPase, Na+/K+ transporting, beta 3 polypeptide | 0.42 |
| OAZ1 | ornithine decarboxylase antizyme 1 | 0.42 |
| RPS19 | ribosomal protein S19 | 0.42 |
| FAM128B | family with sequence similarity 128, member B | 0.41 |
| RPL12 | ribosomal protein L12 | 0.41 |
| RPS3A | ribosomal protein S3A | 0.41 |
| SF3B2 | splicing factor 3b, subunit 2, 145kDa | 0.41 |
| RPS28 | ribosomal protein S28 | 0.41 |
| ANXA2P1 | annexin A2 pseudogene 1 | 0.41 |
| LDHA | lactate dehydrogenase A | 0.41 |
| RPL38 | ribosomal protein L38 | 0.41 |
| RPL37 | ribosomal protein L37 | 0.41 |
| ECEL1 | endothelin converting enzyme-like 1 | 0.41 |
| UQCRH | ubiquinol-cytochrome c reductase hinge protein | 0.41 |
| ENTPD3 | ectonucleoside triphosphate diphosphohydrolase 3 | 0.41 |
| RPL23 | ribosomal protein L23 | 0.41 |
| RPLP0 | ribosomal protein, large, P0 | 0.41 |
| RPL10 | ribosomal protein L10 | 0.40 |
| RPS10 | ribosomal protein S10 | 0.40 |
| RPS2 | ribosomal protein S2 | 0.40 |
| LSS | lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) | 0.40 |

| | | |
|---|---|---|
| CRYBG3 | beta-gamma crystallin domain containing 3 | 0.40 |
| REST | RE1-silencing transcription factor | 0.40 |
| RPL35A | ribosomal protein L35a | 0.40 |
| RPS8 | ribosomal protein S8 | 0.40 |
| RPL21 | ribosomal protein L21 | 0.40 |
| RPL32 | ribosomal protein L32 | 0.40 |
| RPL35 | ribosomal protein L35 | 0.40 |
| RPL26 | ribosomal protein L26 | 0.39 |
| RPSA | ribosomal protein SA | 0.39 |
| RPL37A | ribosomal protein L37a | 0.39 |
| KRTAP17-1 | keratin associated protein 17-1 | 0.39 |
| RPL21 | ribosomal protein L21 | 0.39 |
| RPL3 | ribosomal protein L3 | 0.39 |
| PKM2 | pyruvate kinase, muscle | 0.39 |
| RPS3 | ribosomal protein S3 | 0.39 |
| RRN3P2 | RNA polymerase I transcription factor homolog (S. cerevisiae) pseudogene 2 | 0.39 |
| RPS17 | ribosomal protein S17 | 0.39 |
| SOD1 | superoxide dismutase 1, soluble | 0.39 |
| ANXA2P3 | annexin A2 pseudogene 3 | 0.39 |
| RPL13 | ribosomal protein L13 | 0.39 |
| PPIA | peptidylprolyl isomerase A (cyclophilin A) | 0.39 |
| RPS27 | ribosomal protein S27 | 0.39 |
| RPS29 | ribosomal protein S29 | 0.38 |
| RPS15 | ribosomal protein S15 | 0.38 |
| FTH1 | ferritin, heavy polypeptide 1 | 0.38 |
| OAZ1 | ornithine decarboxylase antizyme 1 | 0.38 |
| FOLR1 | folate receptor 1 (adult) | 0.38 |
| HIC1 | hypermethylated in cancer 1 | 0.38 |
| LOC648771 | similar to 60S ribosomal protein L12 | 0.38 |
| PPP2R1B | protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform | 0.38 |
| RPS15 | ribosomal protein S15 | 0.37 |
| BSPRY | B-box and SPRY domain containing | 0.36 |
| CS | citrate synthase | 0.35 |
| EIF4EBP2 | eukaryotic translation initiation factor 4E binding protein 2 | 0.35 |
| EIF4EBP2 | eukaryotic translation initiation factor 4E binding protein 2 | 0.28 |
| REST | RE1-silencing transcription factor | 0.26 |
| IRF4 | interferon regulatory factor 4 | 0.24 |
| ANKRD30B | ankyrin repeat domain 30B | 0.20 |

# Ⅳ. Discussion

The neuron restrictive silencer factor (NRSF) is a major transcriptional repressor of the neuronal promoter that has a critical role in the regulation of the neuronal gene and a synergic repression effect through its interaction with the other transcription factor (Kim et al., 2004; Kim et al., 2006). NRSF was originally identified as a transcriptional repressor that silenced the neuronal gene in nonneuronal cells. It also acts as a tumor repressor in colon cancer cells (Majumder, 2006), suggesting that NRSF is involved in the signaling pathway in cancer cells and might have a novel function besides transcriptional regulation.

In our previous study, NRSF act as a critical factor to inhibit cell proliferation of KB human oral cancer cells. We found KB cell growth was significantly decreased by NRSF specific target siRNA treatment. Therefore, to understand the NRSE/NRSF regulatory network in KB cells, we performed the microarray analysis by NRSF specific targeted siRNA treatment in KB human oral cancer cells. Microarray data showed that upregulated genes were identified by 117 probe sets, whereas 215 genes were downregulated at least 2-fold, compared with the control (Table 1 and 2). As shown in table 3 and 4, although NRSF has been known as a repressor to silence neuronal gene in nonneurol cells, our data showed numbers of downregulated genes were much more than upregulated genes by NRSF siRNA treatment in KB cells. And also, NRSF protein in KB cells may be involved in a lot of pathway including signal transduction, protein metabolic process, and translational pathway as well as transcriptional regulation pathway.

Several previous studies implied that the NRSF/NRSE (neuron-restrictive silencer element) complex might have a dual function

as a repressor or enhancer, depending on the temporal and spatial context of its expression but provided no solid evidence (Bessis et al., 1997; Kallunki et al., 1998; Seth and Majzoub, 2001). Recent studies reported that NRSF is expressed in neuronal cells and mature neurons as well as in nonneuronal cells (Sun et al., 2005), but its function in these cells remains unknown. However, it is well established that the human genome contains a smaller number of genes than expected. The complexity found in higher organisms can be explained if proteins are multifunctional. Indeed, recent studies continue to reveal proteins that are capable of a broad repertoire of functions (Nedelec et al., 2004). A recent report indicated that the Emx2 homeogene transcription factor is expressed at the mRNA and protein levels in both the nuclear and axonal compartments of olfactory mucosa axon bundles and in axon terminals. Emx2 could interact with the eukaryotic translation initiation factor eIF4E, suggesting a role in translational regulation. TFII-I, which belongs to a family of general transcription factors, is also expressed in both the nucleus and cytoplasm and is involved in signal pathways (Park and Dolmetsch, 2006).

Previous similar study represented a combined bioinformatic and biochemical approach to the genome-wide identification of RE1 sites and REST target genes. They found 1,892, 1,894, and 554 RE1 sites in the human, mouse, and Fugu genomes, respectively. These numbers are considerably greater than those expected by chance alone (770, 653, and 88, respectively). It is important to note that this study, although comprehensive, does not identify all RE1 sites, as exemplified by the secondary RE1 sites discovered in the L1CAM and SNAP25 genes that diverge from the consensus RE1. However, it was considered important to adopt a conservative consensus sequence for this analysis to minimize

identification of false positives. Similarly, adoption of too stringent a consensus could lead to omission of bona fide targets, because individual bases identified as critical in the context of one consensus regulatory element have been shown to be redundant in the context of alternative regulatory elements (Schoenherr et al., 1996; Bulyk et al., 2002). In conclusion, a genome-wide analysis of RE1s and potential REST target genes across three different vertebrate genomes has been undertaken. So We anticipate that membership of these groups will change according to cell type and or developmental stage.

Previous our study showed that NRSF siRNA treatment led to inhibit the cell proliferation and translation. In this data, we could not found cell groth and translational related genes in upregulated genes by NRSF siRNA treated KB cells (Table 3). In contrast, as shown in Table 4, A lot of translational related genes, specially ribosomal genes were downregulated by NRSF siRNA treatment in KB cells. This data indicated that NRSF can mainly control the translational pathway in KB cells.

Originally, NRSF was identified as a repressor that silenced the neuronal gene in nonneuronal cells via binding to the neuronrestrictive silencer element (Kraner et al., 1992; Wood et al., 2003). It has also been reported to function as a tumor repressor mediating the PI(3)K signaling pathway (Schoch et al., 1996), although its exact mechanism of action in this regard remains to be determined. While several reports claimed that in neuronal cell lines, NRSF/NRSE had no regulatory function (Wood et al., 1996), other studies observed that NRSF/NRSE could either increase or decrease expression of its target gene depending on the temporal or spatial context in which the gene was expressed (Wood et al., 1996). In addition, it should be noted that overexpression of the dominant-negative NRSF and knockout of NRSF failed to release

the repression of its gene completely, even though NRSF was originally found as a repressor (Chen et al., 1998). One study in particular reported that a NRSE in the neuronal nicotinic acetylcholine receptor 2−subunit gene may either silence or enhance transcription, depending on the cellular context within the nervous system in transgenic mice. In neuronal cells in vitro, NRSE activates transcription of synthetic promoters when located downstream of the 5′ −untranslated region or at less than 50 bp upstream from the TATA box. However, it switches to a silencer when located further upstream. In contrast, in nonneuronal cells, NRSE always functions as a silencer. Antisense RNA inhibition shows that the NRSEbinding protein NRSF contributes to the activation of transcription in neuronal cells (Bessis et al., 1997). Similarly, in adult neural stem cells, NRSE dsRNA interacted with NRSF resulting in expression of neuronal gene and cell differentiation (Kuwabara et al., 2004)

Although a number of studies have reported that NRSF acts as a transcriptional repressor of over 30 genes and established its mechanism of action, several unsolved issues remain. Why is NRSF expressed in so many neuronal cell types and in mature neurons in brain? Why does it have no apparent function in some cells types? Can NRSF also function as a transcriptional activator? Why are NRSF knockouts unable to fully release its control of gene expression? In this study, we demonstrate one possible function for NRSF in some neuronal cells, not as a transcriptional factor but as a translational activator.

In conclusion, this microarray data and novel putative factors by NRSF expression may provide an insight into the complexity of the regulatory systems involved in the variations of gene expression in different types of the cells.

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# Figure legends

Fig. 1. Visual display of the cluster analysis for NRSF siRNA transfected and control cells.

Fig. 2. Two-dimensinal scatterplot analysis of gene expression values for all genes on the NRSF siRNA transfected KB cells and control cells from the microarray. Red dots represent upregulated genes and green dots represent down-regulated gene at least 2-fold by NRSF siRNA treatment compared with the control.

Fig. 3. Structure of human ribosomal complex and ribosomal related genes. Red stars indicate the down-regulated genes by NRSF siRNA treated in KB cells.

# Figures

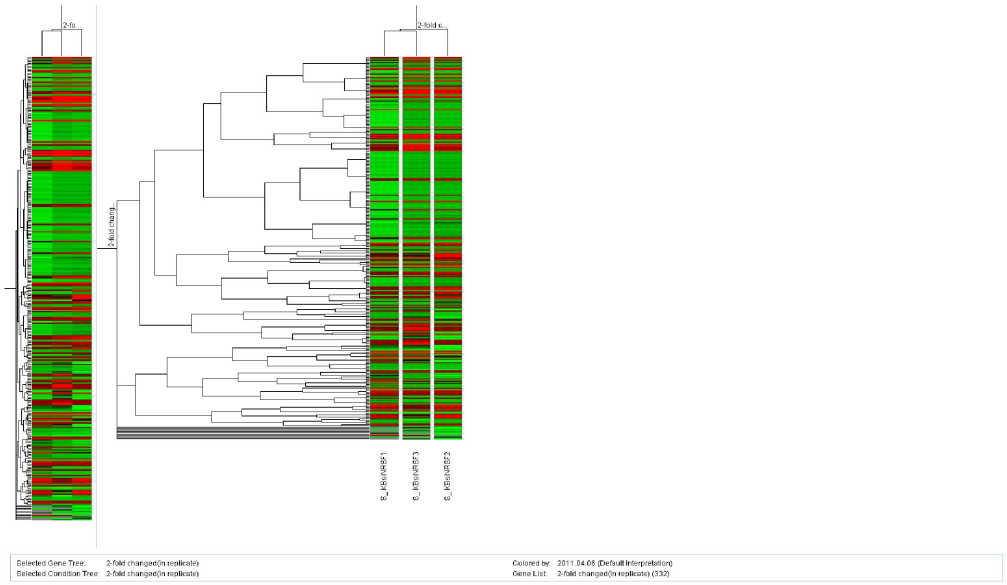


Fig. 1

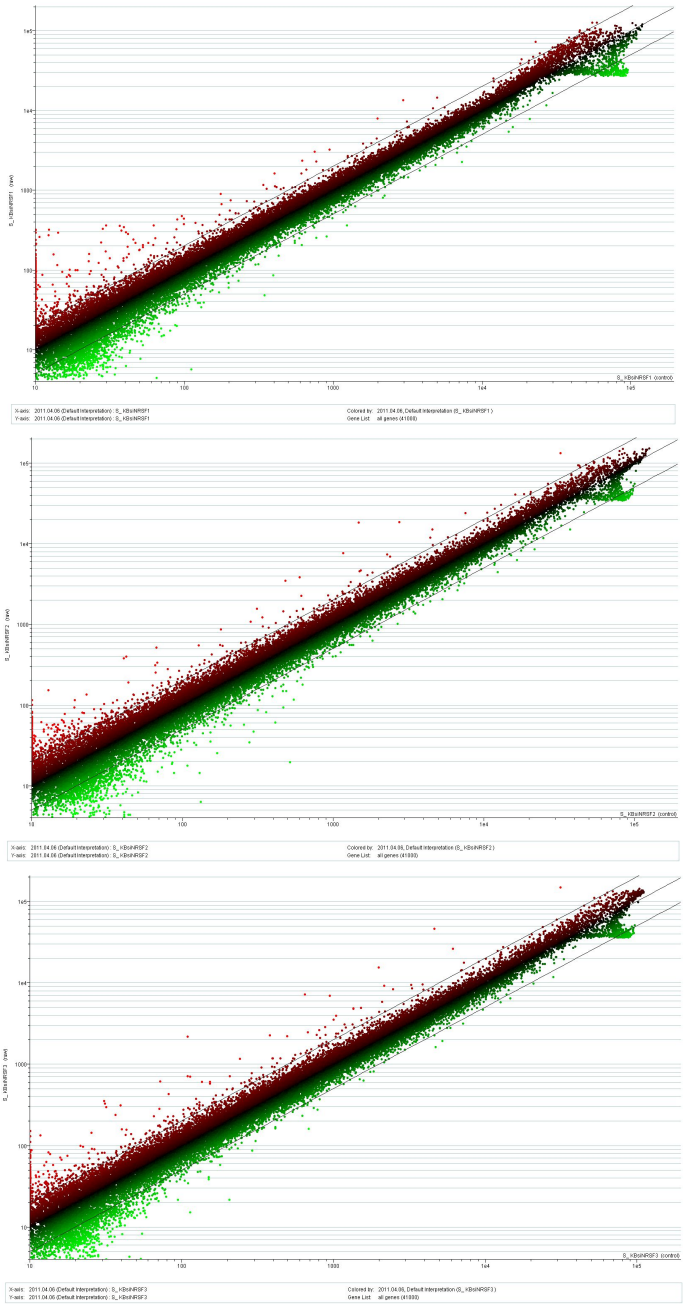

Fig. 2

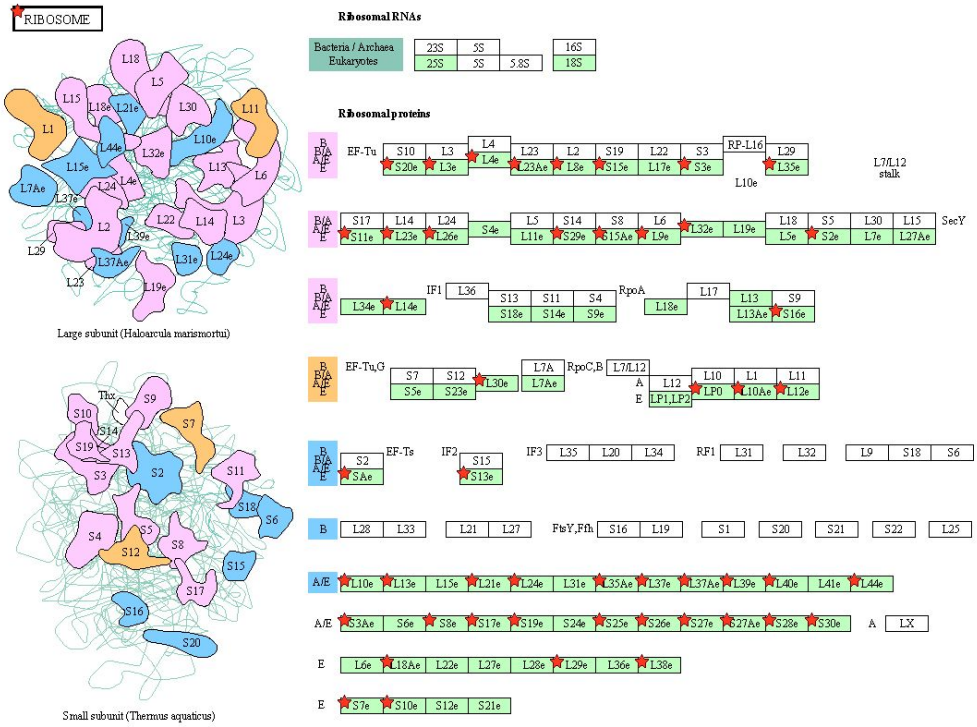
RIBOSOME
Large subunit (Haloarcula marismortui)
Small subunit (Thermus aquaticus)
Ribosomal RNAs
Bacteria / Archaea
Eukaryotes
23S
5S
5.8S
16S
18S
Ribosomal proteins
EF-Tu
RP-L16
L10e
L7/L12 stalk
SecY
IF1
RpoA
EF-Tu,G
L7A
RpoC,B
L7/L12
LP1,LP2
EF-Ts
IF2
IF3
RF1
FtsY,Ffh
A/E
E
A
LX

Fig. 3