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Methods Paper

Novel genes that mediate nuclear respiratory factor 1-regualted neurite outgrowth in neuroblastoma IMR-32 cells

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ABSTRACT

Nuclear respiratory factor-1 (NRF-1) is a transcription factor that functions in neurite outgrowth; however, the genes downstream from NRF-1 that mediate this function remain largely unknown. This study employs a genome-wide analysis approach to identify NRF-1-targeted genes in human neuroblastoma IMR-32 cells. A total of 916 human genes containing the putative NRF-1 response element (NRE) in their promoter regions were identified using a cutoff score determined by results from electrophoretic mobility shift assays (EMSA). Seventy-four NRF-1 target genes were listed according to the typical locations and high conservation of NREs. Fifteen genes, *MAPRE3*, *NPDC1*, *RAB3IP*, *TRAPPC3*, *SMAD5*, *PIP5K1A*, *USP10*, *SPRY4*, *GTF2F2*, *NR1D1*, *SUV39H2*, *SKA3*, *RHOA*, *RAPGEF6*, and *SMAP1* were selected for biological confirmation. EMSA and chromatin immunoprecipitation confirmed that all NREs of these fifteen genes are critical for NRF-1 binding. Quantitative RT-PCR demonstrated that mRNA levels of 12 of these genes are regulated by NRF-1. Overexpression or knockdown of candidate genes demonstrated that *MAPRE3*, *NPDC1*, *SMAD5*, *USP10*, *SPRY4*, *GTF2F2*, *SKA3*, *SMAP1* positively regulated, and *RHOA* and *RAPGEF6* negatively regulated neurite outgrowth. Overall, our data showed that the combination of genome-wide bioinformatic analysis and biological experiments helps to identify the novel NRF-1-regulated genes, which play roles in differentiation of neuroblastoma cells.

1. Introduction

Nuclear respiratory factor-1 (NRF-1) is a major transcription factor in the human genome, which was first known to regulate genes responsible for mitochondrial respiratory function (Scarpulla, 2008) and later found to regulate genes involved in different biological functions such as protein synthesis, signal transduction, organelle biogenesis, cell cycle and cell growth (Efiok and Safer, 2000; Virbasius and Scarpulla, 1994). NRF-1 knockout mice were embryonic lethal, *nrf* mutant zebrafish showed almost absence of photoreceptors, and *ewg* mutant *Drosophila* (the homologue of NRF-1) showed aberrant axonal projection, suggesting that NRF-1 is important for neuronal development (Becker et al., 1998; DeSimone and White, 1993; Huo and Scarpulla, 2001).

We found that NRF-1 functions in neurite outgrowth of neuroblastoma cells (Chang et al., 2005). Overexpression of NRF-1 in human neuroblastoma IMR-32 cells increases the length of the longest neurite. In contrast, dominant-negative NRF-1 decreases neurite length (Chang et al., 2005). We also found that two human genes, *integrin-associated protein* (*IAP*) and *synapsin I* (*SYN1*), are regulated by NRF-1 and mediate its function in neurite outgrowth in IMR-32 cells (Chang and Huang, 2004; Chang et al., 2005; Wang et al., 2009). The NRF-1 response elements (NREs) in the promoter regions of *IAP* and *SYN1* are highly conserved among humans, rats and mice (Chang et al., 2005; Wang et al., 2009). A bioinformatic study based on the consensus sequences predicted that 6% of human promoter sequences contain putative NRF-1 binding sites (FitzGerald et al., 2004). Therefore, we hypothesized that a variety of genes which contain NRE in their promoter regions are involved in NRF-1-regulated neurite outgrowth.

This study adopted a hidden Markov Model (HMM)-based genome-wide approach to search for NRF-1 target genes in the human genome. Bioinformatic software and specific selection criteria were first used to

Abbreviations: NRF-1, nuclear respiratory factor 1; NRE, NRF-1 response element; EMSA, gel electrophoretic mobility shift assays; ChIP, chromatin immunoprecipitation; HMM, hidden Markov Model; shRNA, short hairpin RNA; CDCA7L, cell division cycle associated 7-like; MAPRE3, microtubule-associated protein, RP/EB family, member 3; NPDC1, neural proliferation, differentiation and control, 1; RAB3IP, RAB3A interacting protein; TRAPPC3, trafficking protein particle complex 3; SMAD5, MAD homolog 5 (*Drosophila*); PIP5K1A, phosphatidylinositol-4-phosphate 5-kinase, type I, alpha; USP10, ubiquitin specific peptidase 10; SPRY4, sprouty homolog 4 (*Drosophila*); GTF2F2, general transcription factor IIF, polypeptide 2, 30 kDa; NR1D1, nuclear receptor subfamily 1, group D, member 1; SUV39H2, suppressor of variegation 3–9 homolog 2 (*Drosophila*); SKA3, spindle and kinetochore associated complex subunit 3; RHOA, ras homolog gene family, member A; RAPGEF6, Rap guanine nucleotide exchange factor (GEF) 6; SMAP1, stromal membrane-associated protein 1.

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screen putative NREs in the database of human promoters. Genes containing such NREs were then selected to examine whether their NREs interact with NRF-1, their expression is regulated by NRF-1, and whether they mediate NRF-1-regulated neurite outgrowth in human neuroblastoma IMR-32 cells. Results demonstrate that such an approach is a good strategy to identify novel genes involved in NRF-1-regulated neurite outgrowth in neuroblastoma cells.

2. Materials and methods

2.1. Genome-wide searching for NREs in human promoter sequences

Nineteen known human NRF-1 binding sites were collected according to relevant literature (Supplementary Table S1). The length of the NRF-1 binding site was previously defined as a 12-bp element. To increase searching power, we added five nucleotides upstream and downstream from the 12-bp core element and defined the 22-bp element as NRF-1 response element (NRE). These 19 NREs were aligned by the Clustal W program. The output file was saved as a multiple sequence format (msf).

The HMMER 2.3.2 software, an implementation of the profile Hidden Markov Model (HMM) (Eddy, 1998) downloaded from Washington University (http://hmmer.janelia.org/), was used to perform genome-wide searching for human NREs. The software package includes several programs. Among them, the *hmmbuild* program was used to build an algorithm model of NREs by loading the aligned NRE multiple sequences. The model was then calibrated by the *hummcalibrate* program to increase searching sensitivity. Finally, we used the *hummsearch* program to search for putative NREs. The target queries were 11,699 human promoter sequences, obtained from the database of Transcription Start Site (DBTSS) release 5.0 (Suzuki et al., 2002). The HMMER score was given as the criterion for determining the similarity between the model and target queries. Both Clustal W and the HMMER package were run in a LINUX operating system (Mandrake Corp.).

2.2. Biological classification of genes containing putative NRE

Official symbols of genes with the HMMER score ≧3.5 were submitted to the Generic Gene Ontology (GO) term mapper (http://go.princeton.edu/cgi-bin/GOTermMapper). The parameter of GO slim used was “goa_human_hgnc” (generic GO slim).

2.3. Cell cultures

Human neuroblastoma IMR-32 cells were purchased from the Culture Collection and Research Center, Food Industry and Development Institute, Hsinchu, Taiwan. The cells were grown in minimum essential Eagle's medium with Earle's salt base (Sigma-Aldrich) supplemented with 10% fetal bovine serum (GIBCO). Human embryonic kidney carcinoma 293 T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All cells were cultured at 37 °C in a humidified chamber containing 5% CO_2.

2.4. Electrophoretic mobility shift and supershift assays (EMSA)

EMSA was performed as described previously (Chang and Huang, 2004). For nuclear protein extraction, IMR-32 cells were plated and incubated for 2 days. Cells were collected in cold PBS. The cells were centrifuged at 2000 ×*g* for 2 min, and the supernatant was discarded. The cell pellet was incubated in buffer A on ice for 10 min and then gently shaken for 10 s. The pellet of crude nuclei was collected by centrifugation at 12,000 ×*g* for 10 s. The pellet was re-suspended in buffer C (3 fold volume of pellet) and vortexed for 15 s, and then incubated on ice for 20 min. After centrifugation at 12,000 ×*g* for 2 min, the supernatant containing the nuclear proteins was collected for EMSA and quantified with BCA Protein Assay Reagent (Pierce). Thirty picomoles of each of the forward and reverse oligonucleotides were heated at 94 °C for 2 min and annealed at room temperature for 2 h. The annealed double-stranded oligonucleotides were end-labeled by a fill-in reaction using Klenow DNA polymerase (Promega). The labeled oligonucleotides were purified on Sephadex G-50 columns (Amersham Pharmacia Biotech). The DNA-binding reaction was conducted at 4 °C for 30 min in a mixture containing 3 μg nuclear extracts, 5× DNA shifted buffer, 0.075 μg poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech), and 2×10^4 cpm of ^{32}P-labeled double-stranded oligonucleotides. In supershift assays, antibodies were incubated with the reaction mixture at 4 °C for 3 h before addition of the probes. The anti-NRF-1 goat polyclonal antiserum was kindly provided by Dr. Richard C. Scarpulla. Samples were analyzed on a 4% polyacrylamide gel at 10 V/cm for 2 h.

2.5. Chromatin immunoprecipitation assay (ChIP)

ChIP was performed as described previously (Wang et al., 2009). The ChIP assay was performed with an EZ ChIP™ kit (Millipore Corporation), and conducted following the manufacturer's protocol using mouse IgG, anti-NRF-1 antibodies (Abcam), and not conducting the reactions. The precipitated DNA fragments were amplified by PCR and separated by agarose gel electrophoresis.

2.6. Plasmid constructs

The full-length cDNA of each gene was amplified by PCR using the reverse transcription product obtained from the total RNA of IMR-32 as the template. The amplified cDNA fragments were cut with restriction enzymes and ligated into the linearized pCMS-EGFP vector (Clontech, BD Biosciences). The NRF-1 full-length cDNA was from the previous study (Chang and Huang, 2004). The correctness of each construct was confirmed by DNA sequencing.

2.7. Transient transfection

Transient transfection was performed by the calcium phosphate precipitation method (Jordan et al., 1996). Plasmids were mixed with 0.25 M $CaCl_2$ in 0.1 cy4cx TE buffer. The DNA mixture was mixed with one volume of 2× HEPES buffer and incubated for 1 min at room temperature. The mixture was added to dishes for transfection. After 12 h of transfection, the medium was exchanged with fresh medium, and the cells were grown for further assays.

2.8. Lentiviral production and infection

The recombinant lentiviral production and infection were conducted following the protocol from the National RNAi Core Facility (Academia Sinica, Taiwan). Gene knockdown in IMR-32 cells was achieved using lentiviral transduction to express short hairpin RNA (shRNA) that targets mRNA of *LacZ* (shLacZ, served as control) or the human *NRF-1* (shNRF1) gene. shRNA clones were obtained from the National RNAi Core Facility. The constructs including shLacZ (TRCN0000072226), shNRF1 (TRCN0000016904), shUSP10 (TRCN0000007432), shSPRY4 (TRCN0000056699), shGTF2F2 (TRCN0000028034 7), shNR1D1 (TRCN0000022176), shSKA3 (TRCN0000138582), shRHOA (TRCN0000047711), shRAPGEF6 (TRCN0000047143), and shSMAP1 (TRCN0000153718) were used to generate recombinant lentiviral particles. Human 293T cells were cotransfected with pLKO.1-puro-shRNA, pCMV-deltaR8.91, and pMD.G (two packaging plasmids) using the Lipofectamine 2000 reagent (Invitrogen). Six hours after transfection, the transfection medium was replaced by Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% BSA. After 36 or 60 h, the recombinant lentivirus was harvested. IMR-32 cells were infected at MOI = 3 with 8 μg/ml of polybrene (Sigma-Aldrich). After plating for 1 day, cells were infected by recombinant lentiviruses containing shLacZ, shNRF-1, shUSP10, shSPRY4, shGTF2F2, shNR1D1,