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A genome-wide shRNA screen for new OxPhos related genes

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ABSTRACT

The mitochondrial oxidative phosphorylation (OxPhos) system produces most of the ATP required by the cell. The structural proteins of the OxPhos holoenzymes are well known, but important aspects of their biogenesis and regulation remain to be uncovered and a significant fraction of mitochondrial proteins have yet to be identified.

We have used a high throughput, genome-wide RNA interference (RNAi) approach to identify new OxPhos-related genes. We transduced a mouse fibroblast cell line with a lentiviral-based shRNA-library, and screened the cell population for growth impairment in galactose-based medium, which requires an intact OxPhos system. Candidate genes were ranked according to their co-expression with known genes encoding OxPhos mitochondria-located proteins. For the top ranking candidates the cellular process in which they are involved was evaluated. Our results show that the use of genome-wide RNAi together with screening for deficient growth in galactose medium is a suitable approach to identifying OxPhos-related and cellular energy metabolism-related genes. Interestingly also ubiquitin–proteasome related genes were selected.

1. Introduction

Mitochondria, through the oxidative phosphorylation (OxPhos) system, generate most of the ATP in eukaryotic cells. The OxPhos system is composed of five multisubunit complexes (complexes I to V) located in the inner mitochondrial membrane. These complexes are in turn organized in larger structures or supercomplexes to optimize performance (Acin-Perez et al., 2008). During oxidative phosphorylation, electrons extracted from nutrient compounds are transferred along the electron transport chain (ETC) formed by complexes I to IV. The energy generated through this transfer is used to pump protons across the inner mitochondrial membrane, generating an electrochemical gradient that is ultimately utilized by complex V (ATP synthase) to produce ATP.

The mammalian mitochondrial genome (mtDNA), which is transcribed and translated in the mitochondrial matrix, encodes 13 polypeptide components of the OxPhos complexes (Montoya et al., 2006). These polypeptides are co-assembled with approximately 70 structural subunits encoded by nuclear DNA (Wallace, 2005). The core subunits of the mature complexes are in general well characterized, but the details of their assembly, regulation, and association in different supercomplexes to handle specific physiological situations have not been described (Acin-Perez et al., 2008; Fernandez-Vizarra et al., 2008). The identification of new OxPhos-related proteins will help to understand essential mechanistic details of the OxPhos process, and will also help in the diagnosis of disorders associated with altered OxPhos activity.

Several reports aimed at determining the role of respiratory complex assembly factors (Leary et al., 2007; Vogel et al., 2005; Vogel et al., 2007) and OxPhos structural proteins (Li et al., 2007; Massa et al., 2008) have taken advantage of RNA interference (RNAi) technology. RNAi produces sequence-specific, post-transcriptional gene-silencing, mediated by short (20–25 nucleotides) double-stranded RNA molecules known as silencing RNAs (siRNA), which can be generated in cells by cleavage of longer double-stranded RNAs or short hairpin RNAs (shRNA) (Rao et al., 2009). With whole genome sequences available, massive RNAi screens have become a powerful tool for functional genetic studies (Moffat and Sabatini, 2006). By applying a functional selection to cell populations into which a shRNA library has been introduced, mRNA targets of specific shRNAs can be identified as candidate factors involved in the process under study.

A widely used functional screen for defects in oxidative phosphorylation is metabolic selection in galactose-based culture medium. This was first used for diagnosis in the skin fibroblasts of affected patients (Robinson et al., 1992) and in patient-derived transmitochondrial cell lines (Hofhaus et al., 1996). Later it was used successfully to isolate

Abbreviations: ETC, electron transport chain; OxPhos, oxidative phosphorylation; mtDNA, mitochondrial DNA; RNAi, RNA interference; siRNA, silencing RNA; shRNA, short hairpin RNA; UPS, ubiquitin proteasome system; NER, nucleotide excision repair; RRM, RNA recognition motif.

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OxPhos-compromised cell lines as cellular models of mitochondrial defects (Acin-Perez et al., 2004; Bayona-Bafaluy et al., 2008). Wild-type cells typically grow at near-identical rates in glucose-based medium and medium in which glucose is replaced by galactose. However, whereas in glucose medium much of the ATP derives from glycolysis, in galactose medium the flow of galactose to glucose-1 phosphate is very slow, and consequently most ATP must be obtained from mitochondrial oxidation of pyruvate and glutamine (Reitzer et al., 1979). Cell survival in galactose medium thus depends on a switch from glycolysis to oxidative phosphorylation, which cannot be sustained if an OxPhos gene is silenced. For this reason, cells with defects in mitochondrial oxidative metabolism are at a competitive growth disadvantage when placed in galactose medium, and may be non-viable (Robinson, 1996; Robinson et al., 1992).

Other significant metabolite in OxPhos-impaired cells is uridine, since cellular uracil biosynthesis requires the activity of dihydroorotate dehydrogenase, which in turn needs active respiratory complexes III and IV for its function. Thus, to ensure the viability of OxPhos-impaired cells cultured in glucose medium, uridine supplementation is needed to avoid blockade of endogenous pyrimidine biosynthesis in mutants in which these complexes are affected (King and Attardi, 1989).

The present study combines an array based genome wide RNAi screen with gene co-expression analysis, with the aim of identifying new OxPhos related genes. We designed a loss-of function high-throughput approach that compares growth in glucose medium (supplemented with uridine) versus galactose medium (lacking uridine). Candidate genes were ranked by *in silico* analysis of co-expression with known mitochondria- and OxPhos-related genes. The shRNAs for four candidates that are not involved in expected pathways were further used to experimentally validate the efficiency of siRNA knockdown and their ability to impair growth in galactose medium. We discuss the advantages and pitfalls of the approach and provide important considerations for future studies.

2. Materials and methods

2.1. Cell lines and tissue culture media

The mouse L929$^{Balb/cJ}$ fibroblast cell line is derived from L929 cells in which mitochondria have been replaced with mitochondria from Balb/cJ mice (Bayona-Bafaluy et al., 2003). The L929$^{Balb/cJ}$-K8 line was generated by lentiviral transduction of L929$^{Balb/cJ}$ cells with pLV-tKRAB vector, which encodes constitutive expression of the hybrid protein KRAB (Wiznerowicz et al., 2006). For non-selective culture, all cell lines were grown in DMEM containing 4.5 g/l glucose and 1 mM pyruvate (GibcoBRL), supplemented with 5% FBS (fetal bovine serum, GibcoBRL), uridine (50 μg/ml), 100 IU penicillin and 100 μg/ml streptomycin (Invitrogen). Galactose selective medium was prepared from DMEM medium without glucose or pyruvate (GibcoBRL) by the addition of 0.9 g/l galactose, 110 μg/ml sodium pyruvate, 5% dialyzed FBS, 100 IU penicillin and 100 μg/ml streptomycin (Invitrogen). FBS was dialyzed as described in (Bayona-Bafaluy et al., 2008).

2.2. Experimental design for the shRNA screen

2.2.1. Cell transduction with the shRNA library

Two independent transduction experiments were performed with the GeneNet™ Mouse 40K shRNA Library from SBI System Biosciences (# SI222B-1). The library is pre-packaged in feline immunodeficiency (FIV) viral particles, and was titered for transduction efficiency in the L929$^{Balb/cJ}$ cell line using control VSV-G pseudotyped pFIV-copGFP viral particles carrying GFP. The percentage of fluorescent cells after infection was monitored by fluoresce microscopy. To ensure that cells were not transduced with more than one shRNA, we aimed for a transduction rate below 10%; we therefore used viral particles at a MOI of 0.25, which would infect no more than 7.5% of the cells. An adequate representation after transduction requires that the total number of cells stably transduced be at least 10 times more than the number of shRNAs in the library. Given that fewer than 10% of cells will be transduced, we therefore used 15×10^6 cells, 100 times more than the complexity of the library. Cells, at 70% confluence in 20×100 mm plates, were incubated overnight with medium (3 ml per plate) containing the viral particles and 5 μg/ml polybrene. After 72 h, this medium was replaced with selection medium containing 10 μg/ml puromycin (Sigma), and transduced cells were collected 10 days later. Transduced cells were cultured in glucose or galactose medium for 0, 6 or 15 days.

2.2.2. DNA microarray screening

The siRNA sequences in the shRNA library are selected from among the oligonucleotide probes on the GeneChip® Mouse Genome 430A 2.0 DNA array from Affymetrix (Affymetrix # 900495). To detect siRNA molecules present in cells grown under the two culture conditions (glucose and galactose media at 0, 6 and 15 days), lentiviral inserts containing the siRNA templates were recovered from cell populations according to the manufacturer's instructions and hybridized to the Affymetrix array. Briefly, total RNA was prepared from 9×10^6 cells using the RNA Total Isolation System (Promega). cDNA was synthesized from 1 μg total RNA using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche) and the pFIV-vector specific primer provided with the library. siRNA targets were PCR amplified by nested PCR using biotin-labeled primers and purified with the QUIAGEN PCR purification kit. Purified PCR products (15 μg) were hybridized to the Mouse Genome 430 2.0 Array according to the Affymetrix protocol. All samples were hybridized in duplicate.

2.2.3. Analysis of array data

Each array data set was first normalized using the software provided. Our array data have a heavy tail distribution, and shRNAs with very low abundance will generate low signal-to-noise ratios that preclude statistical analysis. We therefore fixed a threshold by considering only the 25% of shRNAs that gave the strongest hybridization intensities across the arrays in any condition (glucose or galactose culture) and time point. This arbitrary threshold defined 38,488 shRNA molecules that were considered as represented in the transduced cell population (Supplementary Table 1).

To detect shRNA molecules consistently present in cells cultured in glucose medium but not in galactose, only the shRNA molecules that kept hybridization values above the fixed threshold in glucose with the time in culture (6 and 15 days of culture) were considered. shRNA molecules were identified as targeting putative OxPhos genes if they were detected on day 0 and subsequently detected with $\geq$2-fold greater intensity (on average between duplicates) in glucose medium than in galactose on days 6 and 15 in at least one experiment.

2.3. Mitochondrial neighborhood expression analysis

Neighborhood analyses were performed using the GNF mouse Gene Expression Atlas 2 (mouse genome assembly mm9), which contains two replicate datasets for 61 mouse tissues that have been run over Affymetrix expression arrays targeting 36,182 mouse transcripts (Su et al., 2004). From the gnfAtlas2Distance table provided by the UCSC browser (http://genome.ucsc.edu/) we downloaded the distance in expression space from every target gene ($N=438$) to its 1000 closest neighbors. For each target gene we noted all known mitochondria-related and OxPhos genes within its nearest 200 neighbors, referring to these numbers as mt N_{200} and OxP N_{200}, respectively. The average mt N_{200} and OxP N_{200} values were 26 and 2, respectively. The probability of any mRNA corresponding to a mitochondria-related gene is $p=1098/36{,}182=0.03$, and that for