Methods xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Transcriptome guided identification of novel functions of RECQ1 helicase

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ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form 14 April 2016
Accepted 15 April 2016
Available online xxxx

Keywords:
Gene expression
RecQ
Helicase
G4
Cell migration
Cell invasion

ABSTRACT

Gene expression changes in the functional absence of a specific RecQ protein, and how that relates to disease outcomes including cancer predisposition and premature aging in RecQ helicase associated syndromes, are poorly understood. Here we describe detailed experimental strategy for identification of RECQ1-regulated transcriptome that led us to uncover a novel association of RECQ1 in regulation of cancer cell migration and invasion. We initiated a focused study to determine whether RECQ1, the most abundant RecQ protein in humans, alters gene expression and also investigated whether RECQ1 binds with G4 motifs predicted to form G-quadruplex structures in the target gene promoters. Rescue of mRNA expression of select RECQ1-downregulated genes harboring G4 motifs required wild-type RECQ1 helicase. However, some RECQ1-regulated genes are also regulated by BLM and WRN proteins regardless of the presence or absence of G4 motifs. The approach described here is applicable for systematic comparison of gene expression signatures of individual RecQ proteins in isogenic background, and to elucidate their participation in transcription regulation through G-quadruplex recognition and/or resolution. Such strategies might also reveal molecular pathways that drive the pathogenesis of cancer and other diseases in specific RecQ deficiency.

1. Introduction

Compelling evidence from the growing body of work suggests that activities of the *RecQ* gene family may influence carcinogenesis [1–3]. In humans, the RecQ helicase family consists of five known members: RECQ1, BLM, WRN, RecQ4, and RecQ5β. Genetic defects in WRN, BLM and RecQ4 give rise to rare diseases Bloom Syndrome, Werner Syndrome and Rothmund-Thomson/RAPADILINO/ Baller-Gerold Syndromes, respectively. These diseases are each distinguished by clinical features ranging from growth defects (Bloom Syndrome and Rothmund Thomson Syndrome) to premature aging (Werner Syndrome); however, increased genomic instability and predisposition to cancer is a hallmark of RecQ-related diseases [1–3]. Most recently, whole genome sequencing efforts have revealed that mutations in *RECQ1* (also known as *RECQL* or *RECQL1*) gene predispose individuals to familial breast cancer [4,5]. Thus, *RECQ1* is now classified as a breast cancer susceptibility gene. The vast majority of studies on RecQ helicases have focused on investigating their roles in cancer predisposition diseases through DNA repair. In contrast, whether or not these important DNA binding proteins have gene regulatory functions and how that relates to cancer predisposition and other diseases is largely unknown.

Our previous work demonstrated that the transient loss of RECQ1, the most abundant of the five human RecQ proteins, is sufficient to cause genomic instability and reduce proliferation of cancer cells [6–8]. Subsequent studies elucidated that RECQ1 helicase is a major player in maintaining replication fork progression under stress [9–12]. We have found that RECQ1 is enriched at specific genomic regions that pose significant challenge to replication and transcription, and are hotspots for instability and mutagenesis in cancer [12,13]. We reasoned that RECQ1 could be a multifunctional protein due to its high abundance and specific interactions with chromatin. For instance, a RECQ1 homolog in Neurospora mediates posttranscriptional gene silencing [14]. Rat RECQ1 was identified in a piRNA protein complex important for gene silencing [15]. A recent study implicated human RECQ1 in accurate transcription directed by the HomoID box element-containing promoters [16]. To investigate a potential role of human RECQ1 in gene regulation, we initiated a focused analysis of genome-wide changes in gene expression upon RECQ1 knockdown in HeLa (cervical adenocarcinoma) cells that are frequently used to investigate genome stability functions of RECQ1, and highly invasive MDA-MB-231 breast cancer cells which are widely used for molecular and functional analyses of cell migration, invasion and metastasis (Fig. 1) [17].

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http://dx.doi.org/10.1016/j.ymeth.2016.04.018
1046-2023/

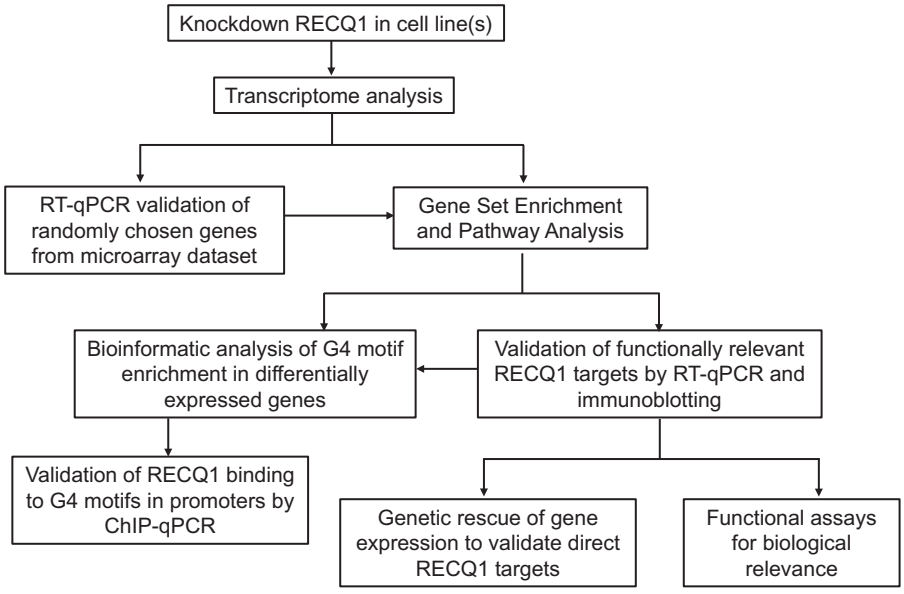

Fig. 1. Experimental approach to determine gene regulatory functions of RECQ1. We utilized siRNA knockdown of RECQ1 and microarray analysis in our original study [17]. Functions of RECQ1 (or other RecQ proteins) can also be investigated following shRNA-mediated knockdown or CRISPR/Cas9-mediated genetic knockout in cell lines. This approach can also be adapted for transcriptome sequencing (RNA-Seq).

2. Description of method

2.1. Cell culture and transfection

HeLa and MDA-MB-231 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Stable shRNA-mediated knockdown of RECQ1 in HeLa and MDA-MB-231 cells was achieved using a lentiviral system as described [9]. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C and routinely checked for mycoplasma contamination (Sigma, catalog no. MP0035). On-Target plus SMARTpool small interfering RNAs (siRNAs) against RECQ1 (NM_032941), WRN (NM_000553), BLM (NM_000057), and non-targeting control (CTL) were purchased from Dharmacon (catalog nos. L-013597-00-0005, L-010378-00-0005, L-007287-00-0005, and D-001810-10-05, respectively). We have previously established the specificity of the siRNA pool [18]. All siRNA transfections were performed by reverse transfection at a final concentration of 20 nM using Lipofectamine RNAiMAX (Invitrogen, catalog no. 13-778-075) as instructed by the manufacturer. For the complementation assays, plasmid transfections for shRNA-resistant RECQ1 expression vectors [9] were performed using Lipofectamine 2000 (Invitrogen, catalog no. 11668019) as instructed by the manufacturer.

2.2. Validation of knockdown by RT-qPCR and immunoblotting

Forty-eight hours after siRNA transfections, total RNA was isolated and whole cell protein lysates were prepared. We consistently observed significant knockdown of *RECQ1* mRNA (>80%) and protein (>75%), as measured by reverse transcription followed by quantitative real-time PCR (RT-qPCR) and immunoblotting. Total RNA from cultured cells was isolated by using Aurum Total RNA Mini Kit (Bio-Rad, catalog no. 7326820) as directed by the manufacturer. For RT-qPCR analysis, 1 μg of total RNA was reverse transcribed using the iScript RT kit (Bio-Rad, catalog no. 1708841), and qPCR was performed using iTaq Universal SYBR Green (Bio-Rad, catalog no. 1725121) as directed by manufacturer. To assess the efficiency of knockdown at the protein level, whole-cell lysates were prepared by using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (Roche, catalog no. 04693132001), and the protein concentration was measured using bicinchoninic acid (BCA) protein quantification kit (Thermo Scientific, catalog no. PI23227). Ten microgram of total protein per lane was loaded on a SDS-PAGE gel and RECQ1 was detected by immunoblotting using anti-RECQ1 antibody (Santa Cruz, catalog no. SC-25547) at 1:1000 dilution. Fifty microgram of total protein per lane was loaded to detect WRN and BLM using anti-WRN (Santa Cruz, catalog no. SC-5629) at 1:500 dilution and anti-BLM (Bethyl Lab, catalog no. A300-110A) antibodies at 1:1000 dilution, respectively. The loading control GAPDH was detected using 1:1000 dilution of anti-GAPDH antibody (Cell Signaling, catalog no. 2118S).

2.3. Transcriptome analysis by microarrays

Forty-eight hour after siRNA transfections in HeLa or MDA-MB-231 cells, total RNA was isolated from triplicate wells of a six well plate using RNeasy minikit (Qiagen, catalog no. 74104). For RNA quality assessment, the RNA integrity number (RIN) was determined for isolated total RNA after running the samples on a Bioanalyzer (Agilent 2100 bioanalyzer). The concentration of the RNA samples was determined using a Nanodrop spectrophotometer (Thermo Scientific). For microarrays, 250 ng total RNA was labeled using Illumina TotalPrep RNA amplification kit (Applied Biosystems, catalog no. 4393543) as recommended by the manufacturer. Microarrays were performed using a HumanHT-12 v4 Expression Bead Chip kit (Illumina, catalog no. BD-103-0204) and analyzed with the R/Bioconductor packages (Lumi.limma). To identify genes differentially expressed upon knockdown of RECQ1 in MDA-MB-231 cells, we used cut-off of 1.42-fold (>30% down-regulation as compared to control siRNA transfected cells) and also analyzed the microarray data based on false discovery rate (adjusted $p < 0.05$). Knockdown of RECQ1 in MDA-MB-231 cells resulted in many more down-regulated genes as compared to up-regulated genes suggesting that RECQ1 may primarily act as a positive regulator of gene expression in MDA-MB-231 cells. Notably, knockdown of RECQ1 did not significantly alter the mRNA levels of other members of the RECQ family including *WRN*, *BLM*, *RecQ4* and *RecQ5β* fur-