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Regulation of Fanconi anemia protein FANCD2 monoubiquitination by miR-302

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ABSTRACT

Fanconi anemia (FA) is a recessively inherited multigene disease characterized by congenital defects, progressive bone marrow failure, and heightened cancer susceptibility. Monoubiquitination of the FA pathway member FANCD2 contributes to the repair of replication stalling DNA lesions. However, cellular regulation of FANCD2 monoubiquitination remains poorly understood. In the present study, we identified the miR-302 cluster as a potential regulator of FANCD2 by bioinformatics analysis. MicroRNAs (miRNAs) are the major posttranscriptional regulators of a wide variety of biological processes, and have been implicated in a number of diseases. Expression of the exogenous miR-302 cluster (without miR-367) reduced FANCD2 monoubiquitination and nuclear foci formation. Furthermore, miR-302 cells showed extensive chromosomal breakage upon MMC treatment when compared to mock control cells. Taken together, our results suggest that overexpression of miR-302 plays a critical role in the regulation of FANCD2 monoubiquitination, resulting in characteristic defects in DNA repair within cells.

1. Introduction

Fanconi anemia (FA) is an autosomal or X-linked recessive genetic disorder associated with bone marrow failure (BMF), congenital abnormalities, and increased susceptibility to cancer, including acute myeloid leukemia [1–3]. The occurrence of FA is estimated to be 1 to 5 per 1,000,000 live births and is caused by biallelic mutation in any one of at least 16 causative genes (FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1, FANCL, FANCM, FANCN/PALB2, FANCP/SLX4, FANCO/RAD51C, FANCQ/ERCC4) [2–7]. Most of these genes, if not all of them, function in critical cellular processes such as DNA replication, cell-cycle control, and the DNA repair mechanism involving DNA interstrand crosslinks through homologous recombination [1,2,4].

Aberrations in the FA pathway lead to inactivation of the multisubunit nuclear complex, the FA core complex, which is comprised of FA proteins such as FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM, and four Fanconi-associated proteins (FAAPs), namely FAAP16, FAAP20, FAAP24, and FAAP100. A central step required to activate the FA core complex is monoubiquitination of FANCD2 and FANCI proteins, which leads to the formation of FANCD2/FANCI heterodimer [2–4,8,9]. Perturbation of the FA core complex causes failure of FANCD2-FANCI monoubiquitination, resulting in abnormal DNA repair signaling. FA is characterized by chromosomal instability and hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC), cisplatin, dipoxybutane, and ionizing radiation. Upon exposure of cells to a DNA crosslinking agent, FANCD2 is monoubiquitinated and relocates to the nuclear DNA damage foci, where it interacts with BRCA1, RAD51, and FANCD1/BRCA2, and is thought to participate in homology-directed DNA repair [10–12]. Thus, monoubiquitination of FANCD2 is a key regulatory step in the response to DNA damaging agents.

It has been reported that FA proteins have several other functions, such as regulation of a family of interferon gamma (IFN-γ)-inducible genes that protect cells against apoptosis [13]. The FA pathway is also involved in cellular antioxidant defense through

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selective protection of antioxidant defense gene promoters [14]; components of the FA core complex interact with HES1 to regulate the Notch signaling pathway [15]. In-depth studies of the functions and developmental aspects of FA genes are very much needed; however, the non-availability of human samples and practical and ethical considerations when working with humans have hampered characterization of FA gene function.

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules about 22 nucleotides in length that tend to bind to target mRNAs at the 3′-untranslated region UTR (3′-UTR) through base pairing. Upon binding, miRNAs regulate the expression level of the target gene by post-transcriptional inhibition, or by inducing degradation [16–20]. miRNAs are involved in diverse processes such as cell development, cell differentiation, cell proliferation, apoptosis, and regulation of protein translation [16–20]. The human miR-302 gene encodes a cluster of nine miRNAs (miR-302a, miR-302a*, miR-302b, miR-302b*, miR-302c, miR-302c*, miR-302d, miR-367 and miR-367*), also termed miR-302s, which are transcribed in a polycistronic fashion [21,22]. miR-302s are specifically expressed in human and mouse embryonic stem cells (ESCs) and human embryonal carcinoma cells (hECCs) [22–25], and this expression is regulated by stem cell marker genes such as Oct3/4, Nanog, Sox2, and Rex1 [24]. Thus, miR-302 is involved in maintenance of totipotency and self-renewal of ESCs. Previous studies demonstrated that miR-302 is also involved in germ layer specialization in early embryonic development [26].

In this study, we investigated the effects of miR-302b, c, a, and d (hereafter referred to as miR-302) expression to test whether the DNA repair mechanism is altered in FA. We demonstrate that miR-302 binds to the 3′ UTR promoter of the FANCD2 gene to regulate gene expression. We demonstrate further that miR-302 is involved in the reduction of FANCD2 monoubiquitination and nuclear foci formation. Finally, we show that miR-302-overexpressing cells undergo extensive chromosomal breakage upon MMC treatment when compared with mock control cells. Taken together, our study results suggest that miR-302 plays a critical role in FANCD2 monoubiquitination, and that expression of miR-302 results in characteristic defects in DNA repair in cells.

2. Materials and methods

2.1. Cell culture

NCCIT (human embryonic carcinoma cell line) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Gibco, Grand Island, NY, USA).

2.2. Plasmid constructs and antibodies

Plasmid construct hsa-miR-302 (b/c/a/d) was kindly provided by Dr. Hal E. Broxmeyer (Indiana University of School of Medicine, USA). EGFP-IRES3-CDHE5 lentiviral vector that has a housekeeping elongation factor 1 alpha promoter (pEF1α) was kindly provided by Dr. Chang Hwan Park (Hanyang University, South Korea). miR-302 cluster was subcloned into the EGFP-IRES3-CDHE5 lentiviral vector, in which EGFP expression is driven by the IRES3 promoter.

A luciferase reporter vector (psiCHECK2; Promega, Madison, WI, USA) was used to generate reporter constructs. A 37-base pair fragments of the wild-type (WT) FANCD2 3′UTR containing the putative miR-302 binding site or a mutant FANCD2 3′ UTR sequence was generated by annealing primer pairs. Fragments were then cloned into the XhoI and NotI sites of the psiCHECK2 vector that contains the *Renilla* luciferase gene as a reporter.

Anti-FANCD2 monoclonal antibody (Abcam, OR, USA) and mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in this study.

2.3. Luciferase assays

The miR-302 target, which contained seed matches, was selected using prediction programs (microRNA.org; http://www.microRNA.org/and TargetScan; http://www.targetscan.org). Wild-type and mutant FANCD2 3′ UTR segments were cloned into the psiCHECK2 vector that contains the *Renilla* luciferase gene as a reporter (Promega). 293T cells were transfected with luciferase reporter plasmid and miR-302. After 48 h of incubation, cells were harvested and assayed using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. Each reporter assay was conducted in triplicate.

2.4. Immunofluorescence microscopy

Immunofluorescence study cells were treated with MMC for 24 h. We followed the cell staining procedure described previously [27]. Anti-FANCD2 monoclonal antibody (1:400 dilution in blocking buffer) was added followed by an overnight incubation at 4 °C, and then secondary CyTM3-conjugated goat anti-mouse antibody (1:300; Jackson ImmunoResearch Laboratories) was added for 1 h in the dark. After mounting with Vectashield (Vector Labs, Burlingame, CA, USA), cell images were acquired using a Deltavision DV Elite microscope system.

2.5. Chromosomal breakage analysis

Cells were treated with 200 nM MMC for 24 h. Following treatment, cells were exposed to colcemid (final concentration of 100 ng/ml) for 90 min, treated with hypotonic solution (0.075 M KCL) for 30 min, and then fixed with 3:1 methanol/acetic acid. Slides were stained with Leishman stain, and 50 metaphase spreads were scored for chromosome breakage.

2.6. Lentiviral vector production and transduction

Lentiviral vectors were introduced into 293T cells with packaging particles by transfection with Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Supernatant fractions were harvested 2 and 3 days after transfection, and stored at 70 °C until use. For viral transduction, NCCIT cells were transduced with viral supernatant containing polybrene (hexadimethrine bromide: 1 μg/ml, (Sigma–Aldrich, St. Louis, MO, USA)).

2.7. Western blot analysis

Proteins were extracted using lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 1% Triton X-100, and 1 mM PMSF). Western blotting analysis was carried out using a monoclonal antibody against FANCD2 (1:1000 dilution). Horseradish peroxidase-conjugated rabbit anti-mouse secondary antibodies were detected by chemiluminescence.

3. Results

3.1. FANCD2 is a direct target of miR-302

It is generally accepted that miRNAs exert their function by regulating expression of their downstream target gene(s). To further explore the molecular mechanism by which miR-302 exerts its biological function, we identified FANCD2 as a potential target of miR-302 because it contains seed matches based on two prediction