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Acute dyskerin depletion triggers cellular senescence and renders osteosarcoma cells resistant to genotoxic stress-induced apoptosis

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ABSTRACT

Dyskerin is a conserved, nucleolar RNA-binding protein implicated in an increasing array of fundamental cellular processes. Germline mutation in the dyskerin gene (*DKC1*) is the cause of X-linked dyskeratosis congenita. Conversely, wild-type dyskerin is overexpressed in sporadic cancers, and high-levels may be associated with poor prognosis. It was previously reported that acute loss of dyskerin function via siRNA-mediated depletion slowed the proliferation of transformed cell lines. However, the mechanisms remained unclear. Using human U2OS osteosarcoma cells, we show that siRNA-mediated dyskerin depletion induced cellular senescence as evidenced by proliferative arrest, senescence-associated heterochromatinization and a senescence-associated molecular profile. Senescence can render cells resistant to apoptosis. Conversely, chromatin relaxation can reverse the repressive effects of senescence-associated heterochromatinization on apoptosis. To this end, genotoxic stress-induced apoptosis was suppressed in dyskerin-depleted cells. In contrast, agents that induce chromatin relaxation, including histone deacetylase inhibitors and the DNA intercalator chloroquine, sensitized dyskerin-depleted cells to apoptosis. Dyskerin is a core component of the telomerase complex and plays an important role in telomere homeostasis. Defective telomere maintenance resulting in premature senescence is thought to primarily underlie the pathogenesis of X-linked DC. Since U2OS cells are telomerase-negative, this leads us to conclude that loss of dyskerin function can also induce cellular senescence via mechanisms independent of telomere shortening.

1. Introduction

Dyskerin is a highly conserved nucleolar protein required for the biosynthesis, maturation, stabilization and function of ribonucleoproteins (RNPs) that incorporate non-coding H/ACA RNAs [1]. H/ACA RNAs include subsets of small nucleolar RNAs (snoRNAs) and Cajal body RNAs, respectively, telomerase RNA (TERC) (which harbors an H/ACA domain at its 3′ end) and at least 350 additional RNAs that have yet to be ascribed specific functions [1,2]. To this end, dyskerin appears to be implicated in an array of fundamental cellular processes. In addition to well-described roles in telomere maintenance [3] and post-transcriptional processing of nascent rRNA [4], dyskerin has also been implicated in regulation of spliceosomal RNA maturation [1], internal ribosome entry site (IRES)-mediated mRNA translation [5], cell proliferation, morphology and adhesion [6–8], mitotic progression [9], and processing of a subset of H/ACA snoRNA-derived microRNAs [10,11]. It is not yet known if dyskerin regulates all of these processes via binding to H/ACA RNAs. Nonetheless, the biologic importance of dyskerin cannot be understated.

Complete dyskerin ablation is lethal in mice, *Drosophila*, and yeast [12]. In humans, germline mutation in the dyskerin gene (*DKC1*) is the cause of X-linked dyskeratosis congenita (DC) [3]. DC is a rare, heritable disorder associated with a wide-ranging and variably severe phenotype, including aplastic anemia, pulmonary fibrosis, cancer susceptibility and signs of premature aging. Telomere dysfunction is thought to primarily underlie the pathogenesis of X-linked DC [4,13]. Through a direct association with TERC, dyskerin plays an important role in telomere homeostasis and maintenance of genomic integrity. Disruption of this interaction impairs telomerase activity, leading to excessive telomere shortening and premature cellular senescence [4,13].

Abbreviations: DC, dyskeratosis congenita; DOXO, doxorubicin; HDACi, histone deacetylase inhibitor; TSA, trichostatin A; CQ, chloroquine; SAH, senescence-associated heterochromatinization.

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Although dyskerin mutation may increase cancer susceptibility, wild-type dyskerin and/or its mRNA are overexpressed in various sporadic cancer types, and high-levels may be associated with poor prognosis [14–16]. We and others previously demonstrated that acute loss of dyskerin function via siRNA-mediated depletion slowed the proliferation of transformed human cell lines [6,7]. However, the mechanism remained unclear.

Described herein, we show that targeted dyskerin depletion induced the senescence of U2OS osteosarcoma cells as evidenced by proliferative arrest, senescence-associated heterochromatinization, and a senescence-associated global gene expression profile. Chromatin compaction can promote cell survival, and this can be reversed through the use of agents that induce chromatin relaxation [17–19]. To this end, dyskerin-depleted cells were resistant to apoptosis induced by genotoxic stress, whereas agents that induce chromatin relaxation sensitized the cells to apoptosis. U2OS cells are telomerase-negative and do not express either TERC or telomerase reverse transcriptase [6]. This leads us to conclude that loss of dyskerin can induce cellular senescence via mechanisms independent of telomere dysfunction.

2. Materials and methods

2.1. Cell culture

U2OS cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's minimal essential medium with GlutaMAX (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum with 100 IU/mL penicillin and 100 IU/mL streptomycin (Invitrogen). Quality control was sporadically performed using the Mycoplasma Plus™ PCR Primer Set (Agilent Technologies, Santa Clara, CA).

2.2. siRNA transfections

Custom-designed (#1) and pre-designed (#2) ON-TARGETplus SMARTPool siRNA duplexes targeting *DKC1* and negative control, ON-TARGETplus Non-Targeting siRNA pools #1 and #2 were obtained from Dharmacon (Lafayette, CO). The siRNA sequences are listed in Table S1. Transfections were performed using Lipofectamine 2000 (Invitrogen), as previously described [6].

2.3. Chemical reagents and antibodies

Doxorubicin, neocarzinostatin, trichostatin A, suberoylanilide hydroxamic acid (SAHA), chloroquine and bafilomycin were obtained from Sigma–Aldrich (St. Louis, MO). Antibodies recognizing dyskerin and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies, including secondary antibodies, were obtained from Cell Signaling Technology (Danvers, MA).

2.4. Protein extraction and immunoblotting

Protein extractions and Western blots were performed as previously described [6]. In most cases, the blots were stripped and re-probed with a different antibody.

2.5. Cell proliferation and apoptosis analyses

Proliferation was assessed by incubating the cells with 10 μM 5-ethynyl-2′-deoxyuridine (EdU) for 16 h and then analyzed using the Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (Invitrogen) as per the manufacturer's protocol. Apoptosis was measured using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen), as previously described [9]. Analyses were performed on a BD™ LSR II flow cytometer (BD Biosciences, Sparks, MD) and tabulated using FlowJo Version 10 (Tree Star, Ashland, OR). Statistical analyses were performed using Student's *t*-test; $p < 0.05$ was considered statistically significant.

2.6. Indirect immunofluorescence and analysis

Cells were grown and transfected in 4-well Lab Tek chamber slides (Thermo Scientific, Rochester, NY). At the indicated time points, the cells were fixed, permeabilized, immunolabeled, and analyzed as previously described [9].

2.7. RNA extraction and analysis

Total RNA was isolated using the miRNeasy kit (Qiagen, Valencia, CA) as per the manufacturer's protocol. The miScript PCR system (Qiagen) was used for quantitative RT-PCR on a 7500 Real Time PCR System (Applied Biosystems, Carlsbad, California). All Quantitect primers were obtained from Qiagen. Gene expression profiling was performed using the GeneChip Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA). The associated technical work and bioinformatics analyses were conducted by the University of Pennsylvania Molecular Profiling Core.

3. Results

Almost all of the studies described herein were performed over a 72–144 h time frame, depending on the specific experiment. Thus we reasoned that any observed phenotype associated with dyskerin depletion would not be a result of accelerated telomere shortening. To further ensure this, we employed telomerase-negative U2OS cells which express relatively high levels of dyskerin [6,15]. After 72 h, dyskerin expression was reduced more than 80–90% relative to the controls irrespective of the siRNA used (Fig. S1).

3.1. Loss of dyskerin function arrests cell proliferation

Forty-eight hours after siRNA transfection there were no appreciable differences in EdU incorporation between the control (siCTRL) and dyskerin-depleted (siDKC1) cells (Fig. 1A). After 72 h siCTRL cells continued to incorporate EdU, whereas siDKC1 cells showed no additional uptake ($p < 0.0001$). Cells transfected with two distinct *DKC1* siRNAs (siDKC1 #1 and siDKC1 #2) yielded similar results. This reaffirmed our previous findings that acute loss of dyskerin function arrested U2OS proliferation [6]. EdU incorporation was reduced in a relatively equal proportion of siCTRL and siDKC1 cells following 24 h treatment with the genotoxic agent doxorubicin (DOXO) (Fig. 1A). In addition to no appreciable differences in apoptosis between untreated siCTRL and siDKC1 cells (see Fig. 3A), this confirmed that siDKC1 cells were still viable and responsive to genotoxic stress.

3.2. Autophagy is not induced by dyskerin depletion

We and others previously noted that dyskerin-depleted cells exhibited unusual morphology whereby their cytoplasmic processes were thinned and markedly elongated [6,7]. As soon as 72 h but more prominently 6 days after transfection, there was also cytoplasmic vacuolization with most of the vacuoles arranged in a perinuclear pattern (Fig. 1B). The vacuoles were qualitatively larger than those seen in siCTRL and GAPDH knockdown cells.

The vacuolization was reminiscent of the autophagosomes that form during the course of autophagy. Autophagic flux as measured by accumulation of the LC3B cleavage product (LC3B II) is considered a reliable measure of autophagy; LC3B II is recruited to

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