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Ploidy-dependent change in cyclin D2 expression and sensitization to cdk4/6 inhibition in human somatic haploid cells☆

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ABSTRACT

Near-haploidy is observed in certain cancer types, but ploidy-dependent alterations in gene regulation in the haploid state remain elusive. Here, by comparative transcriptome analysis between human isogenic haploid and diploid cell lines, we found lowering of cyclin D2 level in haploids. Acute genome duplication in haploids restored cyclin D2 expression to diploid level, indicating that the regulation of cyclin D2 expression is directly linked to ploidy. Downstream pathways of cyclin D2, such as Rb phosphorylation and p27 sequestration remained intact in haploids, suggesting that they adapt to lowered cyclin D level. Interestingly, however, haploid cells were more susceptible to cdk4/6 inhibition compared to diploids. Our finding indicates feasibility of selective growth suppression of haploid cells based on ploidy-linked gene regulation.

1. Introduction

Alterations in chromosome number are hallmark of cancer cells. Tetraploidy, the doubling of the whole chromosome sets of normal diploid cells, is observed in broad spectrum of cancers [1–5]. Artificially-induced tetraploidization has been demonstrated to promote tumorigenesis in rodents, indicating important pathological contribution of tetraploid cells to malignancy [6,7]. On the other hand, albeit with much lower frequency compared to tetraploidy, near-haploidy is also observed in certain types of blood and solid cancers, being regarded as signs of poor prognosis [8–17]. Near haploid somatic cells are generally very unstable and easily convert to diploids both in vitro and in vivo [18,19]. Once diploidized, they are nearly indistinguishable from canonical diploid cells by conventional chromosome diagnoses [8,20]. Therefore, it is possible that much more cancer cell types than those currently recognized have passed through “near-haploid phase” in their history.

A comprehensive understanding of ploidy-dependent alteration in gene regulation would provide important information for developing new cancer therapeutic strategies that enable selectively targeting of cancer cells with abnormal ploidy states. Recent comparative transcriptome and proteome analyses between human isogenic diploid and tetraploid cells revealed that expression of G1/S cyclin, cyclin D is commonly upregulated in several tetraploid cell lines [21,22]. Cyclin D mediates entry into the cell cycle through activation of its binding partners cyclin-dependent kinase 4/6 (cdk4/6) [23], and was proposed to be required for overriding p53-mediated suppression of proliferation upon tetraploidization. Another proteome study reported tetraploidy-linked activation of mitotic regulatory pathways, which was proposed to overcome mitotic stresses arose from increased number of chromosomes upon tetraploidization [24]. Moreover, several comparative pharmacological studies between diploid and tetraploid cell lines have revealed selective growth suppression of tetraploid cells by mitosis- or cell cycle-related cytotoxic compounds [25–27]. On the other hand, while recent studies revealed haploidy-linked p53 upregulation or mitotic stress arising from haploidy-specific centrosome loss, it remains largely unknown how haploidy effects global gene regulation [28,29]. It also remains to be determined whether there are any molecular targets with which near-haploid cancer cells are selectively affected. In this study, we performed comparative transcriptome analyses between isogenic haploid and diploid human cells, through which we identified

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haploidy-linked alterations in expression of several genes including reduction of cyclin D2. Based on these results, we found haploid cells are more susceptible to cdk4/6 inhibition compared to their diploid counterparts.

2. Materials and methods

2.1. Cell culture, and flow cytometry

The haploid HAP1 cells were maintained as previously described [28]. Diploid and tetraploid HAP1 cell lines were established as previously described [28]. For DNA content analyses, 2×10^6 cells were stained with 10 μg/mL Hoechst 33342 (Dojindo) for 15 min at 37 °C, and fluorescence intensity was analyzed using a JSAN desktop cell sorter (Bay bioscience). Cell synchronization was performed as previously described with modifications [30]. Shortly, asynchronous cells were treated with 20 ng/mL nocodazole for 4 h, and washed with culture medium three times. Mitotic cells were shaken off and subjected to immunoblotting at each time point indicated.

2.2. Cell proliferation assay

For cell proliferation assay, cells were seeded on 96-well plates at 9,000, 4,500, or 2250 cells/well (for haploid, diploid, or tetraploid HAP1 cells, respectively). After 24 h, cells were treated with different concentrations of PD0332991 (PZ0199, Sigma-Aldrich), LY2835219 (HY-16297, MedChemExpress) or doxorubicin hydrochloride (040–21521, Wako). Forty-eight h after addition of the compounds, 5% Cell Counting Kit-8 (Dojindo) was added to culture medium, incubated for 4 h, and absorbance at 450 nm was measured using the Sunrise plate reader (Tecan).

2.3. RNAi

The siRNA sequences used in this study are 5′-CGAUGCCUCUUUGAAUAAA-3' (Anillin) [31], and 5′-CGUACGCGGAAUACUUCGA-3' (Luciferase). siRNA transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific).

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as previously described [32]. For signal detection, the ezWestLumi plus ECL Substrate (ATTO) and a LuminoGraph II chemiluminescent imaging system (ATTO) were used. Quantification of CBB staining or immunoblotting signals was performed using the Gels tool in ImageJ software (National Institutes of Health).

2.5. RNA-seq and differentially expressed gene (DEG) analysis

Total RNA was isolated from asynchronous cell culture using NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instruction. Library preparation, sequencing and analysis were performed by Macrogen Inc. (Seoul Korea) as previously described [33]. Briefly, integrity of total RNA was checked using an Agilent 2100 Bioanalyzer. cDNA libraries were constructed using TruSeq RNA Sample Prep Kit v2 (Illumina), and quantified using 2100 Bioanalyzer. One hundred-base paired end sequencing was conducted on the Illumina HiSeq 2000. Overall reads' quality, total bases, total reads, GC (%) and basic statistics were calculated by FastQC program version 0.10.0, and adapter sequences and low quality reads removed by Trimmomatic program version 0.32. The trimmed reads were mapped to UCSC hg19 human genome with HopHat version 2.0.13. Then, -G option of Cufflinks version 2.2.1 was used to assemble transcripts from aligned reads and calculate expression profiles of assembled transcripts. Expression profiles were expressed as the fragments per kilobase of transcript per million mapped reads (FPKM). To facilitate the statistical analysis with a balanced data distribution, 1 was added to the raw signals (FPKMs) and transformed the data to log 2. After log transformation, in order to reduce systematic bias, quantile normalization was used with preprocessCore' R library. Statistical analysis was performed using fold change per comparison pair. The significant results were selected on conditions of $|fc| \geq 2$.

2.6. Quantitative real-time PCR

Reverse transcription reactions were conducted using 1500 ng of total RNA template in 60 μl of total volume reactions using SuperScript VILO master mix (Thermo Fisher). The reaction solutions were diluted 5 time in nuclease-free water, and real-time PCR was performed on the Light Cycler 480 (Roche) in 20 μl reaction mixture containing 5 μl cDNA template, 500 nM forward and reverse primers, and 100 nM universal probe (Roche). Primers used for qRT-PCR were listed in Table S1.

2.7. Bromodeoxyuridine (BrdU) incorporation assay

BrdU incorporation was conducted as previously described [28]. Chromosomes were stained using 1.0 μg/mL DAPI (Dojindo). Fixed cells were observed under a TE2000 microscope (Nikon) equipped with a × 40 0.95 NA Plan Apo objective lens (Nikon), and an ORCA-ER CCD camera (Hamamatsu Photonics). Image acquisition was controlled by μManager software (Open Imaging).

2.8. Antibodies

Antibodies used in this study were listed in Table S2. Fluorescence- or horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and used at dilution of 1:1000 for IF and IB.

3. Results and discussion

3.1. Ploidy-dependent changes in gene expression between haploid and diploid HAP1 cells

To gain insight into haploidy-linked changes in gene expression profiles, we performed comprehensive next generation RNA-seq using near-haploid human somatic cell line, HAP1 and its isogenic diploid counterpart (Fig. 1A) [28,34]. Among ~70 genes indicated to be up- or downregulated in haploid cells compared to diploids in differentially expressed gene analysis (Fig. 1B, and Table S3), we confirmed the ploidy-dependent changes in transcriptional and translational levels of several genes by quantitative RT-PCR (for GPC3/glypican3, IGFBP5, CCND2/Cyclin D2, ANXA1, and LAMB1) and immunoblotting (for glypican3, and cyclin D2), respectively (Fig. 1C and D). Of interest, glypican3 or cyclin D2, whose expression is reportedly downregulated or upregulated, respectively, in the tetraploid state compared to diploid counterparts in different types of cells [21,35], was found to be upregulated or downregulated, respectively, in haploid HAP1 cells. These results indicate general linearity in ploidy-dependent changes in regulation of certain genes across hypo- and hyperdiploid states. Cell cycle distribution was similar between asynchronous haploid and diploid cells (Fig. 1A), ruling out the possibility that the ploidy-dependent change in cyclin D2 expression is merely an indirect consequence of altered cell cycle distribution between different ploidies. Moreover, the ploidy-dependent change in cyclin D2