Regulation of Cancer Cell Proliferation by R273H p53 and RNF4

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Abstract

R273H p53 is a gain-of-function mutant p53 reported to increase cell proliferation (Iwanaga and Jeang, 2002) and the transcription of proteins necessary for DNA replication (Polotskaia et al., 2015). The abnormally high rate of proliferation of cancer cells increase the amount of replication stress experienced by the cell. RNF4 is a homolog of Slx5/Slx8 that may share the function of the Slx5/Slx8 heterodimer in ubiquitinating mitotic checkpoint proteins (Thu et al., 2016) to allow cellular replication to proceed under unideal conditions, such as replication stress. Thus, it seems possible that the increased cell proliferation due to R273H p53 may be maintained via RNF4, where R273H p53 increases replication stress while RNF4 alleviates it. We hypothesize that the co-overexpression of R273H p53 and RNF4 would have the greatest effect in increasing cell proliferation, compared to the overexpression of WT p53 and RNF4. The cell proliferation rate of cancer cell line MCF-7 have been quantified through BrdU and MTS assay. We report that the BrdU assay was unable to be an appropriate measurement due to the lack of differentiation between control and experimental conditions, and that the results of the MTS assay suggests that the overexpression of RNF4 on top of p53, WT or R273H, decreases cell proliferation.

Introduction

Replication stress is a cellular phenomenon in which normal DNA replication isstalled or compromised due to an increase in malfunctioning replication forks (Horejsí et al., 2005). If unalleviated, replication stress may damage the genome, evoking a DNA damage response leading to senescence or apoptosis (Hills and Diffley, 2014). This is particularly beneficial in inhibiting the uncontrolled proliferation of tumor cells. Alternative defense mechanisms against replication stress have been highlighted. The Slx5/Slx8 heterodimer in *S. cerevisiae* targets mitotic checkpoint proteins for degradation and allows DNA replication to progress under replication stress (Thu et al., 2016). RNF4, an E3 SUMO-targeted ubiquitin ligase (STUbL), is a homolog of Slx5/Slx8 in mammals. Deletion of the RNF4 gene increases sensitivity to DNA damaging agents in human cells (Yin et al., 2012) and RNF4 has been observed to be recruited to sites of DNA damage (Galanty et al., 2012), implicating the protein in maintaining genome stability.

The vandalization of genome integrity is a common characteristic in cancer cells, which accrue mutations to perpetuate abnormal cell proliferation. Although the DNA damage repair functions of RNF4 have been studied, its function in cancer cells is yet to be determined. As a homolog of Slx5/Slx8, RNF4 may also alleviate replication stress in mammalian cells by targeting checkpoint proteins for degradation. Previously, we overexpressed RNF4 in breast cancer cell lines MCF-7 and MDA-MB-468, and observed that the frequency of mitotic cells decreases for MDA-MB-468 and increases for MCF-7 cells. This result suggests that RNF4 may alleviate replication stress in MDA-MB-468 but not in MCF-7. The cause of this discrepancy may be due to genetic differences between these two cell lines: MDA-MB-468 harbors a gain-of-function mutant R273H p53 while MCF-7 carries WT p53. As cell lines carrying WT p53 are expected to enter G2 arrest under replication stress (Nitta et al., 2004), the mutant p53 in MDA-MB-468 may be failing to share the function of its WT and induce mitotic arrest instead. Furthermore, the induced mitotic arrest

may be escaped by the overexpression of RNF4, which may share the function of Slx5/Slx8 and ubiquitinate the mitotic checkpoint proteins (Thu et al., 2016). These observations suggest the possibility of cross-talk between p53 and RNF4 in regulating the mitotic checkpoint.

MDA-MB-468's R273H p53 may be functionally similar to another gain-of-function mutant 281G p53, which has been known to increase the transcription hsMad1, which promotes abnormal cell proliferation (Iwanaga and Jeang, 2002). This increase in proliferation would increase the amount of replication stress experienced by the cell. Consequently, cells with the R273H mutation would be more affected by the overexpression of RNF4, if RNF4 is able to alleviate replication stress. Furthermore, previous studies have implicated R273H p53 as a regulator of PARP1, an enzyme critical for DNA replication and repair, and showed that cells expressing R273H p53 increase transcription of DNA replication proteins PCNA and MCMs (Polotskaia et al., 2015). Therefore, both R273H p53 and RNF4 seem to possess functions which support cancer cell survival. We hypothesize that MCF-7 cells transfected with R273H p53 and RNF4 will display greater cell proliferation than those transfected with WT p53 and RNF4. The rate of proliferation have been attempted to be quantified through BrdU (5-bromo-2'-deoxyuridine) assay and fluorescence microscopy to detect the incorporation of BrdU in newly synthesized DNA. However, the assay proved to be ineffective, and an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay was performed instead. The MTS assay utilizes NADH/NADPH produced by viable cells to reduce the MTS tetrazolium compound into a colored formazan product, which is soluble in tissue culture. The absorbance of media can be read to determine the amount of product produced, which correlates to the rate of proliferation. We found that the overexpression of WT or R273H p53 increases proliferation, but the subsequent overexpression of RNF4 decreases proliferation.

Results

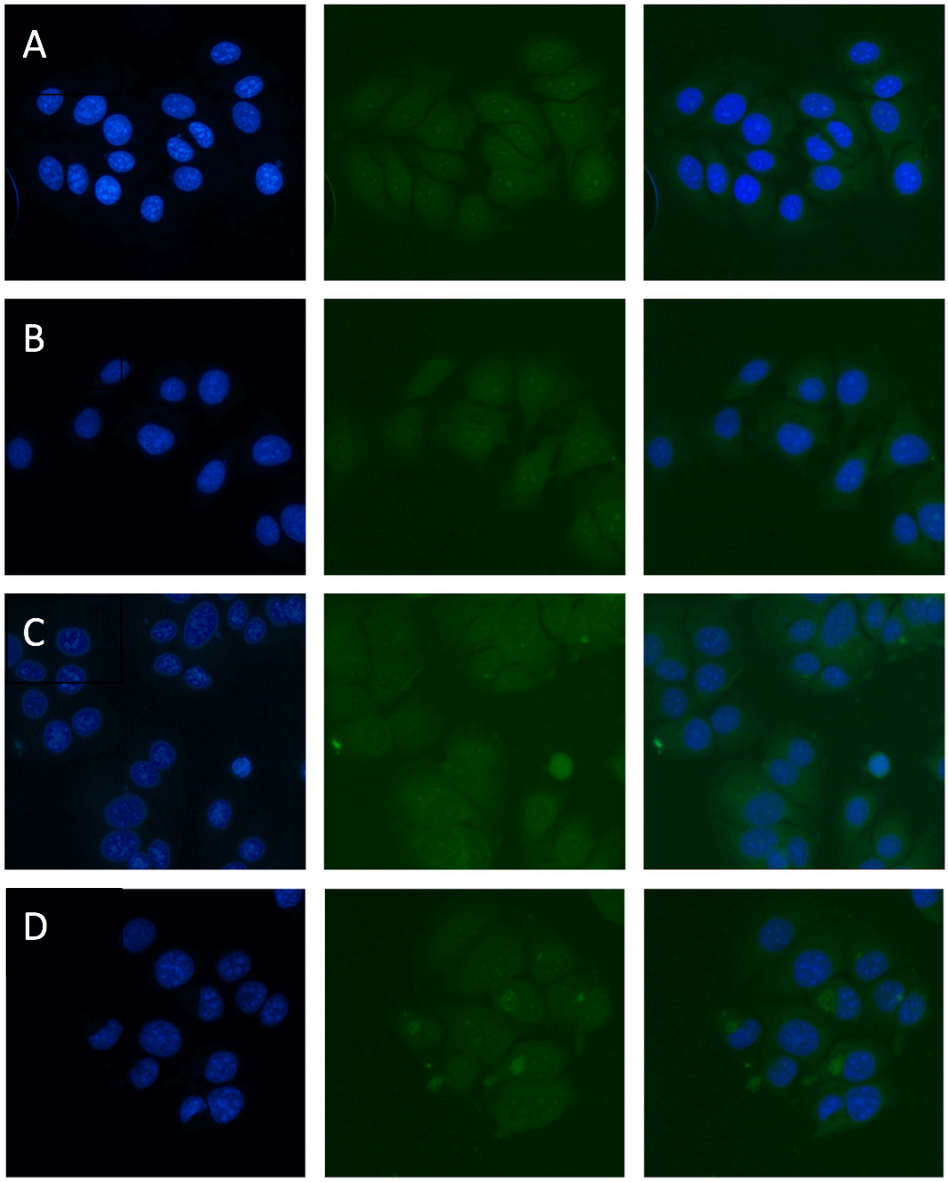


Figure 1. No difference in BrdU detection between negative control and experimental MCF-7 cells. MCF-7 cells were incubated in BrdU incorporation and antibody labeling solution for 2 hours each unless stated otherwise. Images were taken under FITC channel to detect BrdU antibody fluorescence. Blue stain indicates DAPI, green stain indicates BrdU. A) Negative control: Complete growth media + no incubation in BrdU labeling solution. B) No serum in growth media + BrdU incubation. C) Complete growth media + BrdU incubation. D) Same conditions as (C) but with overnight antibody labeling.

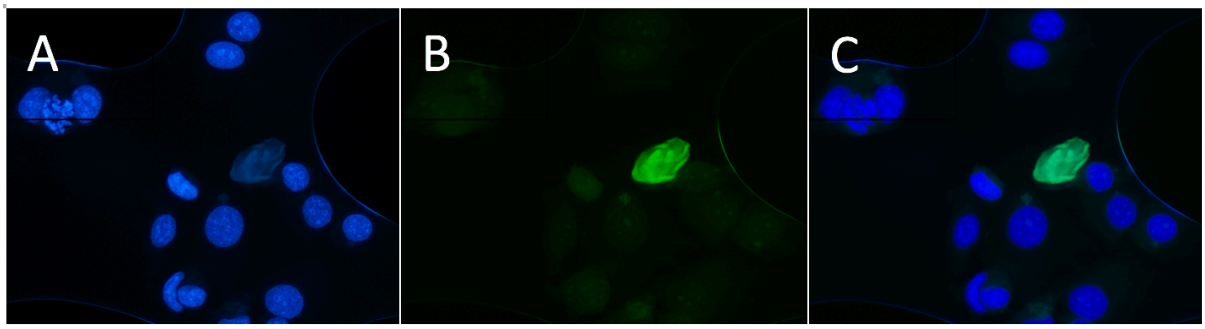
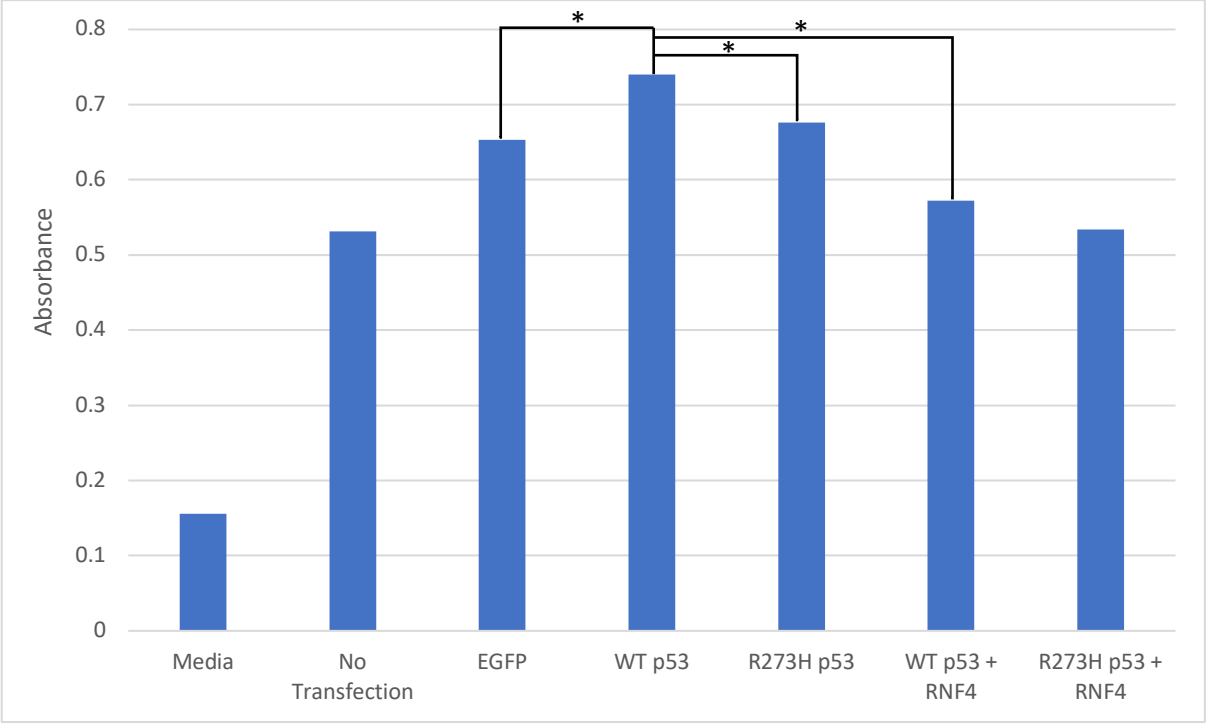


Figure 2. Only debris fluoresce in the BrdU assay.
MCF-7 cells were incubated in BrdU incorporation and antibody labeling solution. Coverslips were mounted using DAPI. Only cell debris was found to fluoresce under FITC channel used to detect BrdU antibody. A) Cells visualized by DAPI. B) Cells visualized by BrdU antibody. C) Overlap of A and B.



** significant at $p < 0.1$.

Figure 3. Overexpression of RNF4 under both WT p53 and R273H p53 overexpression seems to decrease cell proliferation. MCF-7 cells were transfected for 48 hours and treated with MTS reagent for 1 hour. Absorbance was read at 490 nm. Condition (mean, St. dev): Media (0.156, 0.047), No Transfection (0.531, 0.103), EGFP (0.653, 0.174), WT p53 (0.74, 0.236), R273H p53 (0.676, 0.198), WT p53 + RNF4 (0.572, 0.086), R273H p53 + RNF4 (0.534, 0.081). n = 6.

BrdU Assay fails to assess cell proliferation.
MCF-7 cells were labeled with BrdU. Two negative controls were conducted: one without serum in growth media to inhibit replication, and another without incubation in BrdU labeling solution. Our results show that BrdU incorporation cannot be successfully determined in comparison to cells that have not been labeled with BrdU (Fig. 1) and that the only fluorescence detected pertained to cellular debris (Fig. 2). Under these circumstances, the BrdU assay cannot act as an accurate read out for measure cell proliferation. Another trial increased the BrdU incorporation period up to three hours and the antibody labeling period to last overnight, but no significant differences between experimental conditions were observed (Fig. 1).

Co-overexpression of RNF4 with either WT or R273H p53 decreases cell proliferation.
MCF-7 cells were separately transfected with EGFP, WT p53, R273H p53, WT p53 and RNF4, and R273H p53 and RNF4. Post-transfection, cells were treated with MTS for 1 hour and had their absorbance read at 490 nm. EGFP transfections gave an absorbance of 0.653, 23.0% greater than the absorbance of cells without any transfections (Fig. 3). WT p53 and R273H p53 transfections gave an absorbance of 0.74 and 0.676, respectively, both greater than that of EGFP (Fig. 3). Simultaneous transfections of p53 and RNF4 gave absorbances of 0.572 and 0.534 for WT p53 and R273H p53, respectively (Fig. 3). The absorbance of WT p53 + RNF4 was 22.7% less than that of WT p53 transfections, and the absorbance of R273H p53 + RNF4 was 21.0% less than that of R273H transfections.

Discussion

BrdU detection via fluorescent imaging does not measure cell proliferation.
As shown in results, the BrdU assay is inadequate to measure cell proliferation and fails to even differentiate between MCF-7 cells that have received BrdU and those that have not (Fig. 1). We have tried multiple times to troubleshoot this observation. Extension of the BrdU antibody labeling incubation period did not have an effect, nor did increasing the time of BrdU incorporation. Unfortunately we had to conclude that the BrdU assay was no longer suitable for this experiment and chose to conduct the MTS assay instead.

Overexpression of WT p53 increases cell proliferation, but overexpression of R273H does not.
MCF-7 cells were transfected and treated with MTS reagent to measure cell proliferation via absorbance. Transfections with WT p53 had an absorbance of 0.74, 13.3% greater than the transfection control using EGFP transfections (Fig. 3). On the other hand, R273H p53 transfections gave an absorbance of 0.676, only 3.52% greater than that of EGFP (Fig. 3). This result contradicts the findings of Iwanaga & Jeang (2002), who showed that R273H p53 should increase cell proliferation via the increased transcription of hsMad1. As both experiments utilized transfections, the discrepancy is likely to be due to experimental error. Nitta et al. (2004) also showed that cell lines carrying WT p53 enter G2 arrest under replication stress, which should not increase cell proliferation as seen in our experiment.

Co-overexpression of WT p53 and RNF4 decreases cell proliferation.
Transfection of WT p53 + RNF4 gave an absorbance of 0.572, 22.7% less than that of the single WT p53 transfection (Fig. 3). No statistical significance could be found between the absorbance of R273H and R273H p53 + RNF4 transfections, but the same trend of decreased proliferation could be seen (Fig. 3). These results go against our hypothesis that MCF-7 cells transfected with R273H p53 + RNF4 would have a greater proliferation rate than cells transfected with WT p53 + RNF4. Both R273H p53 and RNF4 possess functions that

contribute to cancer cell survival. R273H p53 increases the transcription of proteins crucial for DNA replication (Polotskaia et al., 2015), and RNF4 is involved in DNA damage responses maintaining genome integrity, and potentially alleviating replication stress as a homolog of Slx5/Slx8 (Yin et al., 2012; Galanty et al., 2012; Thu et al., 2016). For these reasons we have hypothesized the possibility of cross-talk between R273H p53 and RNF4, where R273H p53 increases cell proliferation and consequently, replication stress, and RNF4 alleviating the increased replication stress to allow abnormal proliferation to continue. Our results show, however, that the overexpression of both WT and R273H p53 have opposite effects to the overexpression of RNF4, where p53 transfections increase proliferation and the corresponding RNF4 transfections decrease proliferation (Fig. 3). Perhaps the ubiquitin ligase functions of RNF4 are not acting upon mitotic checkpoint proteins to allow proliferation to continue, but upon another cellular mechanism that leads to decreased cell viability. This would explain the decrease in absorbance under RNF4 transfections (Fig. 3). However, as the experiment did not contain single RNF4 transfections in MCF-7, the isolated impact of the overexpression of RNF4 is unknown, but can be of focus in future experiments.

Methods

Miniprep of pRK5 plasmid:

Procedure was adapted from Wizard®Plus SV Minipreps DNA Purification System. Plasmid purchased as transformed bacteria. Incubated in 37°C shaker for 18 hours in 5mL L-Broth including 100µg/mL ampicillin. Overnight culture was pelleted by centrifugation. Cells were resuspended, lysed, and treated with Alkaline Protease before neutralized. Plasmid DNA was isolated by binding to spin column, washed, and eluted with nuclease-free water. RNF4 was isolated from the pRK5 plasmid. WT p53 and R273H p53 were isolated from ____ and ____ plasmids, respectively.

Transfection of MCF-7.

All cells were cultured in 37°C/5% CO_2 incubator. Cells were grown on cover slips in DMEM +10% fetal bovine serum + antibiotic/antimycotic drugs (complete media). Transfection procedures were taken from *Lipofectamine™ 3000 Reagent Protocol*. Volumes of reagents used were indicated in the 6-well and 96-well column. Transfected cells were incubated for 48 hours.

BrdU Assay

Post-transfection in a 6-well plate, cells were incubated with 10 µM BrdU in complete media for 2-3 hours. Cells were then washed with 1X PBS (3 X 2 min), fixed in 3.7% formaldehyde in 1X PBS (15 min), treated with permeabilization buffer (0.1% Invitrogen Triton X-100 in 1X PBS) (20 min), 1 N HCl (10 min), 2 N HCl (10 min), phosphate/citric acid buffer (0.2 M Na_2PO_4, 0.1 M Citric Acid, pH 7.4) (10 min). Cells were incubated for 2 hours or overnight in Alexa Fluor 488 antibody staining buffer (1X PBS, 0.1% Triton X-100, 5% FBS) and washed in permeabilization buffer. Coverslips were mounted by SlowFade Diamond Antifade DAPI on to a glass slide and sealed by nail polish the next day. Glass slides were stored in the dark at 4°C until examined by fluorescent microscopy under the DAPI and FITC channel.

MTS Assay

Post-transfection in a 96-well plate, cells were treated with the CellTiter 96® AQ_{ueous} One Solution Reagent ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS[(a)]] and phenazine ethosulfate; PES) for 1 hour. Absorbance was read at 490 nm using a 96-well plate reader.

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