Inactivating Mutations in Exonuclease and Polymerase Domains in DNA Polymerase Delta Alter Sensitivities to Inhibitors of dNTP Synthesis

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POLD1 encodes the catalytic subunit of DNA polymerase delta (Pol δ), the major lagging strand polymerase, which also participates in DNA repair. Mutations affecting the exonuclease domain increase the risk of various cancers, while mutations that change the polymerase active site cause a progeroid syndrome called mandibular hypoplasia, deafness, progeroid features, and lipodystrophy (MDPL) syndrome. We generated a set of catalytic subunit of human telomerase (hTERT)-immortalized human fibroblasts expressing wild-type or mutant *POLD1* using the retroviral LXS vector system. In the resulting cell lines, expression of endogenous *POLD1* was suppressed in favor of the recombinant *POLD1*. The siRNA screening of DNA damage-related genes revealed that fibroblasts expressing D316H and S605del *POLD1* were more sensitive to knockdowns of ribonuclease reductase (RNR) components, RRM1 and RRM2 in the presence of hydroxyurea (HU), an RNR inhibitor. On the contrary, SAMHD1 siRNA, which increases the concentration of dNTPs, increased growth of wild type, D316H, and S605del *POLD1* fibroblasts. Hypersensitivity to dNTP synthesis inhibition in *POLD1* mutant lines was confirmed using gemcitabine. Our finding is consistent with the notion that reduced dNTP concentration negatively affects the cell growth of hTERT fibroblasts expressing exonuclease and polymerase mutant *POLD1*.

Keywords: polymerase delta, mutation, siRNA screening, fibroblasts, human

Introduction

THE ESSENTIAL *POLD1* GENE encodes the catalytic subunit of one of the main replicative DNA polymerase delta (Pol δ) (Rayner *et al.*, 2016). Its fidelity derives from a highly selective polymerase active site coupled to a proof-reading exonuclease domain that edits nucleotide mis-insertions at the primer end. The primary role of Pol δ is in lagging strand synthesis during replication, but it also functions as the main gap-filling polymerase during DNA repair. During polymerization, the template and primer are held by the palm, finger, thumb, and exonuclease domains, while N-terminal amino domain interacts with unpaired segment of the template strand (Fig. 1) (Swan *et al.*, 2009).

A number of somatic mutations that alter the exonuclease domain have been reported in various types of cancers (Palles *et al.*, 2013; Bellido *et al.*, 2016; Rayner *et al.*, 2016). Several germline mutations in the exonuclease domain coding sequence are also known to predispose to can-

cers, mainly familial colorectal cancers (Palles *et al.*, 2013; Bellido *et al.*, 2016). In contrast to these oncogenic mutations, other germline mutations of the *POLD1* gene cause an autosomal dominant disorder known as the mandibular hypoplasia, deafness, progeroid features, and lipodystrophy (MDPL) syndrome (mandibular hypoplasia, deafness, progeroid features, lipodystrophy) (Weedon *et al.*, 2013; Pelosini *et al.*, 2014; Lessel *et al.*, 2015; Fiorillo *et al.*, 2018). A recurrent heterozygous *POLD1* mutation, p.Ser605del, identified in MDPL patients is located in the highly conserved motif A of the polymerase active site involved in the alignment of the incoming complementary dNTP with the primer terminus (Swan *et al.*, 2009). *In silico* analysis predicted that p.Ser605del mutant polymerase would be able to bind DNA, but not catalyze polymerization (Weedon *et al.*, 2013). Biochemical studies of recombinant *POLD1* protein with p.Ser605del showed no detectable polymerase activity, while retaining lower but considerable exonuclease activity (Weedon *et al.*, 2013). The MDPL patients show various

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FIG. 1. Structural domains of Pol δ and the locations of POLD1 mutations. The structure of the Pol δ catalytic subunit from *Saccharomyces cerevisiae* (Swan *et al.*, 2009) is depicted as ribbon diagram. The palm (purple) finger (blue) and thumb (green) domains as well as exonucleolytic proofreading domain (red) surround template strand (dark red) and primer (yellow). The polymerase active site is located within the palm domain where dNTP (green) is shown. Locations of the D316H and S605del changes are indicated by labeled yellow spheres. Pol δ , polymerase delta. Color images are available online.

features of accelerated aging, but cancer risk does not appear to be increased (Lessel *et al.*, 2015). The p.Arg507Cys substitution located within the exonuclease domain has been reported in a MDPL patient (Pelosini *et al.*, 2014). The p.Arg507Cys substitution is distant from the exonuclease active site and may compromise polymerase function by allowing the primer strand to slip out and induce replication stress, as proposed for p.Ser605del (Lessel *et al.*, 2015).

To date, there is no treatment for MDPL syndrome. In an attempt to identify potential therapeutic targets, we conducted high-throughput siRNA screening of fibroblasts carrying the POLD1 disease mutants. We did not find any siRNA that diminished the cellular disease phenotypes of the POLD1 mutant lines. We did, however, find a differential response of POLD1 mutant fibroblasts to siRNAs and reagents known to alter dNTP synthesis. Notably, siRNAs targeting SAMHD1, a dNTPase, improved cell proliferation and viability, suggesting that increasing dNTPs may be a potential therapeutic modality to explore in the future.

Materials and Methods

Establishment of cell lines expressing wild-type and mutant POLD1

Immortalized human diploid fibroblast lines, 82-6pBlox, expressing wild-type and mutant POLD1 were established as described previously (Huang *et al.*, 2008). The full length POLD1 cDNA was generated from the 82-6pBlox cell line by iProof HF PCR Kit (cat. no. 172-5310, Bio-Rad) and was subcloned into pLXSN vector. An exonuclease mutation, c.947G>C, p.D316H, and a polymerase mutation, c.1812_

1814del, p.S605del, were introduced by PCR-based site directed mutagenesis using iProof HF polymerase. Following the viral production in TSA54 cell line and retrovirus infection, cultures were selected with 250 μ g/mL G418 for 7 days. These cell lines were maintained in DMEM with 10% fetal calf serum (FCS) and 1 $\times$ penicillin/streptomycin in 5% CO₂ and 5% O₂ atmosphere. During the siRNA screening, cell lines were kept in the 5% CO₂ incubator with ambient oxygen.

DNA sequencing and Western analysis

Sanger sequencing of PCR and RT-PCR products of the POLD1 gene in expression constructs and established cell lines were performed as previously described (Lessel *et al.*, 2015). Western analysis of POLD1 protein was performed with commercially obtained anti-POLD1 antibody (dilution 1:200, clone A9, cat. no. sc-17776, Santa Cruz Biotechnology) and biotinylated anti-mouse antibody (cat. no. BA9200, Vector Laboratories).

High-throughput siRNA screening

The siRNA library screening was performed at the University of Washington Quellos High-Throughput Screening Core using the previously published DDR322 siRNA library (Kehrli *et al.*, 2016). The DDR 322 siRNA library consists of commercially obtained three independent siRNAs that target each of 322 genes involved in double strand DNA break repair (cat. no. 1027416, Qiagen, Germantown, MD) (Kehrli *et al.*, 2016). For siRNA screening, 200 cells were plated per well in 384-well plates, and 24 h later, cells were transfected with the siRNA library using DharmaFECT1 (cat. no. T-2001, Dharmacon, Lafayette, CO) (Kehrli *et al.*, 2016). Transfections of three independent siRNAs per gene per well were done in triplicate with a mock control and universal siRNA control in each plate. Transfection efficacy was monitored by the toxicity of Kif11 siRNA controls placed in each plate. 24 h later, hydroxyurea was added at final concentrations of 1 or 2 mM. Two days later, relative cell numbers were determined by the CellTiter-Glo Assay (cat. no. G7570, Promega, Madison, WI) according to the manufacturer's instructions. The signal of siRNA-transfected wells were normalized to mock transfected wells to obtain the relative cell number. Student's *t*-test was performed to test mean by well and treatment/vehicle mean by well and treatment. Pairwise *p*-values were calculated using test triplicates versus vehicle triplicates.

Gemcitabine and hydroxyurea sensitivity assay

For hydroxyurea sensitivity assays, 2000 cells per well were plated in 24-well plates in presence of the specified amount of hydroxyurea (HU). Forty-eight hours later, fractions of live and dead cells were determined by Cell Viability Imaging Assay (cat. no. R10477, InVitrogen) (Johnson *et al.*, 2013).

For gemcitabine assays, 500 cells per well were plated in 96-well plates in duplicate (controls in quadruplicate) in presence of specified concentrations of gemcitabine. Forty-eight hours later, the CellTiter-Glo Assay was performed according to the manufacturer's instructions. Relative cell numbers of treated wells were normalized to those of

untreated wells. Statistical significance was determined by Student's *t*-test.

Results

Establishment of fibroblast lines carrying *POLD1* mutations

To conduct siRNA screening for cellular pathways that may influence the severity of *POLD1* diseases, full length wild-type or mutant *POLD1* cDNA were introduced into the catalytic subunit of human telomerase (hTERT) immortalized human fibroblast cell line, 82-6pBlox, via the LXSXN retroviral system (Miller and Rosman, 1989). In LXSXN system, *POLD1* is driven by the retroviral long terminal repeat (LTR) promoter. We chose to engineer the mutations into fibroblasts, since previous studies showed that patient derived fibroblasts carrying *POLD1* mutant alleles exhibited disease phenotypes, characterized by nuclear anomalies, micronuclei, altered cell growth, cellular senescence, and defects in DNA damage repair (Fiorillo *et al.*, 2018). We generated three mutant *POLD1* constructs for these studies. One construct carried the S605del mutation, a recurrent mutation of MDPL syndrome (Lessel *et al.*, 2015; Bellido *et al.*, 2016) (Fig. 1). The second encoded a D316H substitution in the exonuclease domain, reported in a large pedigree of early onset gastrointestinal cancers (Bellido *et al.*, 2016). D316H alters one of the catalytic carboxylates of the exonuclease domain (Fig. 1) and had previously been demonstrated to increase mutation rate in yeast when engineered at the corresponding position (Kokoska *et al.*, 2000; Murphy *et al.*, 2006). The exonuclease mutant was included in this study because we speculated that siRNAs that are able to correct cellular disease phenotypes of S605del but not D316H would be more specific as a therapeutic candidate of the MDLP syndrome. Moreover, siRNA screening might identify pathways that can be targeted for the treatment of *POLD1* associated cancers, as reported in mismatch repair deficient cancers (Chan *et al.*, 2019; Lieb *et al.*, 2019). The third construct was engineered with both D316H and S605del mutations (DM). The purpose of the double mutant, although not seen in human disease, was to assess whether siRNAs that affect both of the single mutants do so by acting by a similar mechanism. In this case, the phenotype of the double mutant cell line may be similar to

one or both of the single mutants. Alternatively, if a siRNA affects the *POLD1* alleles by different mechanisms, the phenotype of the double mutant may be additive.

Following the establishment of stable cell lines, the presence of the transgenes and their mutations was confirmed by PCR, followed by Sanger sequencing (data not shown). Western analysis of the *POLD1* protein showed that *POLD1* expression was slightly increased in wild type (WT) and D316H mutant cells compared to the parent cell line (120% and 111%, respectively) and reduced in S605del and DM mutant cells (51% and 63%, respectively), suggesting that S605del reduces the stability of the *POLD1* protein (Fig. 2A). RT-PCR sequencing revealed that the *POLD1* mRNAs expressed in D316H, S605del, and DM *POLD1* lines were all derived from the LXSXN-*POLD1* transgenes (Fig. 2B), indicating that endogenous *POLD1* expression in mutant *POLD1* lines was suppressed in these lines. The *POLD1* mRNA expressed in untransfected 82-6pBlox and WT *POLD1* lines were all wild-type sequence. We were unable to amplify the intronic regions of *POLD1* or intronic regions of two other genes, namely *WRN* and *LMNA*, using the same RT-PCR templates, excluding the possibility of genomic DNA contamination in RT-PCR template. The same phenomenon was observed in a different cell line, 88-1pBlox, transformed with the same LXSXN-*POLD1* S605del construct (data not shown). This suggests that *POLD1* expression levels may be tightly regulated for optimal cell growth and/or survival of hTERT fibroblasts when introduced through LXSXN. When *POLD1* cDNAs were introduced through the pLOC lentiviral system, endogenous *POLD1* continued to be expressed along with the over-expressed *POLD1* transgene (data not shown), indicating that endogenous *POLD1* promoter suppression does not always occur in *POLD1* cDNA transfected cells. The benefit of this unexpected transcriptional regulation with the LTR promoter, as demonstrated by western blotting, is that the mutant *POLD1* proteins are expressed at levels comparable to WT *POLD1* in nontransformed cells.

Hypersensitivity to dNTP synthesis inhibitors in *POLD1* mutant cell lines

We initially characterized the sensitivity of the cell lines to genotoxic agents, based on previous observations that human *POLD1* knockdown and mutant cells are sensitive to

FIG. 2. The human hTERT fibroblast lines expressing wild-type and mutant *POLD1*. (A) Western analysis of *POLD1* cDNA expressing hTERT lines. Lamin A/C is shown as the loading control. (B) RT-PCR sequencing of *POLD1* cDNA expressing lines. The presence of mutations at amino acid positions 316 and 605 is shown. hTERT, catalytic subunit of human telomerase; WT, wild type. Color images are available online.

HU (Tumini *et al.*, 2016; Fiorillo *et al.*, 2018) as are WRN-deficient cells (Poot *et al.*, 2001; Ammazalorso *et al.*, 2010). The *WRN* gene, null mutations at which are responsible for Werner syndrome, encodes a nuclease helicase that cooperates with POLD1 in maintaining genomic stability (Kamath-Loeb *et al.*, 2000; Szekely *et al.*, 2000). Twenty-four hour HU treatment caused a significant reduction in the live-to-dead cell ratio in POLD1 mutant cells in a dose-dependent manner, as assessed by an imaging-based cell viability assay (Fig. 3A). At 1 mM HU, there was a trend toward lower viability in the mutant lines that was not statistically significant. However, at 10 mM HU, the viability of the 82-6pBlox parent cell line (90.6%) and WT POLD1 control line (93.9%) were significantly higher than that of lines expressing the D316H (73.8%), S605del (74.5%), or DM (51.2%) alleles. At 20 mM HU, viability of the single mutant cell lines decreased even further (D316H, 60.2%; S605del, 54.8%; DM, 60.2%). HU impairs cell growth by targeting ribonucleoside diphosphate reductase (RNR) (Mathews, 2015). Thus, both mutant *POLD1* alleles likely enhance the sensitivity of cells to reductions in dNTP pools. Since the double mutant cell line showed enhanced HU-sensitivity at 10 μ M HU, relative to the single mutant lines, the alleles likely cause sensitivity by slightly different mechanisms.

We then conducted a siRNA library screening to identify modifiers of HU hypersensitivity that may differentially alter the HU sensitivity among cells carrying wild-type POLD1, D316H mutant, and S605del mutant lines. The previously described DDR322 siRNA library (Kehrli *et al.*, 2016), which contains 966 siRNAs targeting 322 genes implicated in DNA repair, was chosen to focus on the known mechanisms of POLD1 mutation and HU. Due to the setup of the siRNA screening facility, the screening was done in 20% ambient oxygen instead of 5% low oxygen applied in the rest of the study. HU concentrations of 1 and 2 mM were chosen for this screening based on the preliminary study showing the wide spread of modifier siRNAs in both suppressor and enhancer directions at these concentrations.

Data analysis of siRNA HU modifier screening revealed RRM2 siRNA as a modifier that differentially affected POLD1 mutants and controls (Table 1). The *RRM2* gene encodes the small subunit and rate limiting component of RNR, which is a tetrameric enzyme composed of homodimers of RRM1 (the catalytic subunit) and RRM2 (Aye *et al.*, 2015). The RRM2 siRNA inhibited the cell growth of the D316H line to 56.0% (HU 1 mM) and 69.8% (HU 2 mM) relative to the untransfected control. RRM2 siRNA also suppressed growth of S605del line to 75.1% (HU 1 mM) and 84.3% (HU 2 mM) while it did not affect HU toxicity of WT-POLD1 line. RRM1 siRNA decreased relative cell numbers of S605del line to 80.3% (HU 1 mM) and 65.7% (HU 2 mM), but did not appear to modify HU toxicity in WT and D316H lines (Table 1). Another component that influences dNTP pools emerged as an enhancer of cell growth. SAMHD1 is a dNTP triphosphohydrolase that negatively regulates nuclear dNTP concentration. SAMHD1 siRNA, which results in the increase of dNTP concentration (Lahouassa *et al.*, 2012), increased cell numbers 167% (HU 1 mM) and 225% (HU 2 mM) in the WT culture, 151% (HU 1 mM) and 145% (HU 2 mM) in the D316H line, and 176% (HU 1 mM) and 173% (HU 2 mM) in the S605del line

FIG. 3. Sensitivities of cell lines with WT and mutant POLD1 to HU and gemcitabine. (A) Fraction of dead (gray) and live (black) cells are shown following 48 h HU treatments of indicated concentrations. * $p < 0.05$. (B) Relative cell numbers following the 24 h gemcitabine treatments are shown with increasing concentrations. (C) Statistical significance data at 1 μ M concentrations of gemcitabine (B) are shown. HU, hydroxyurea.

(Table 1). None of the DDR322 siRNA library components enhanced the growth of the S605del cell line without also enhancing growth of the D316H and WT-POLD1 lines.

To confirm the inhibitory effect of RRM1 siRNA and RRM2 siRNA on cell growth in mutant POLD1 lines, we

TABLE 1. MODIFIERS OF HYDROXYUREA TOXICITY IN POLD1 EXPRESSING hTERT FIBROBLASTS

<i>POLD1</i> genotype	<i>WT</i>		<i>D316H</i>		<i>S605del</i>	
HU concentration (mM)	1	2	1	2	1	2
RRM1 siRNA						
Test/vehicle ratio	0.862	1.058	0.829	0.935	0.803	0.657
Pairwise <i>p</i> -value	0.463	0.787	0.125	0.235	0.023	0.002
RRM2 siRNA						
Test/vehicle ratio	0.942	1.238	0.560	0.698	0.751	0.863
Pairwise <i>p</i> -value	0.638	0.083	0.001	0.027	0.019	0.067
SAMHD1 siRNA						
Test/vehicle ratio	1.670	2.250	1.515	1.448	1.756	1.727
Pairwise <i>p</i> -value	0.037	0.021	0.013	0.006	0.001	0.092

Relative cell numbers in siRNA transfected cells (test) and control (vehicle) in presence of 1 or 2 mM HU concentrations are shown. Test/vehicle ratios are averages of triplicates, where higher than 1 indicates the siRNA improves cell viability in presence of HU, and less than 1, that the siRNA increases HU toxicity. Pairwise *p*-values are calculated from the triplicate sets. Bold numbers indicate statistical significance of 0.05. See text for details.

hTERT, catalytic subunit of human telomerase; HU, hydroxyurea; WT, wild type.

examined the effect of gemcitabine, a cytidine analog commonly used in cancer therapy. The diphosphorylated form is a potent RNR inhibitor (Heinemann *et al.*, 1990; Wang *et al.*, 2007), while the triphosphorylated form functions as a chain terminator in DNA synthesis (Rizzuto *et al.*, 2017). After 48 h gemcitabine treatment at a concentration above 1 μ M, viability of D316H, S605del, and double mutants were significantly lower than parent 82-6pBlox or wild-type POLD1 lines, as determined by CellTiter-Glo assay (Fig. 3B). At 1 μ M gemcitabine, we observed a decrease in viability to $60.0\% \pm 2.3\%$ for 82-6pBlox and $59.3\% \pm 3.1\%$ for WT controls, compared to $36.8\% \pm 2.0\%$ for D316H, $37.8\% \pm 2.5\%$ for S603del, and $35.0\% \pm 4.4\%$ for DM (Fig. 3C). Combined with the results of HU and siRNA against RRM1 and RRM2, we conclude that hTERT fibroblasts expressing exonuclease or polymerase mutant POLD1 are hypersensitive to the reagents known to reduce dNTP concentrations.

Discussion

The MDPL syndrome is a child-onset progeroid syndrome caused by a recurrent mutation in the polymerase domain of POLD1 (Weedon *et al.*, 2013; Lessel *et al.*, 2015). Fibroblasts derived from the MDPL syndrome exhibited accelerated cellular senescence phenotypes and a persistence of DNA damage after the exposure to cisplatin, an alkylating agent (Fiorillo *et al.*, 2018). In this study, we attempted to identify siRNAs that ameliorate the cellular disease phenotypes of POLD1 mutant fibroblasts with the eventual goal of identifying potential interventions, with a special interest in p.Ser605del mutation. Current siRNA library screening using DDR siRNA library failed to reveal such siRNA candidates. Instead, we observed that D316H, S605del, and DM POLD1 lines were equally sensitive to siRNA and drugs that reduce dNTP pools.

RNR performs the rate limiting step in the generation of dNTP pools, which are necessary for DNA replication and repair processes (Aye *et al.*, 2015). The siRNA screening and a further confirmation study showed that siRNAs against the two components of RNR, RRM1, and RRM2, as well as

gemcitabine differentially effect POLD1 mutants compared to the controls. A recent study of human mammary epithelial cells demonstrated that genetic or pharmacological inhibition of RRM2 promotes replicative senescence (Delfarah *et al.*, 2019), supporting the role of dNTP pools in cellular senescence. The S605del is located in the motif A region involved in binding of the dNTP substrate (Swan *et al.*, 2009; Weedon *et al.*, 2013), which explains our observation that expression of this mutant led to higher sensitivity to reduction in dNTP levels. This is supported by rescue with the siRNA depletion of the SAMHD1 dNTP triphosphohydrolase, which elevates dNTP pools (Coquel *et al.*, 2018). It is puzzling that both the exonuclease and the polymerase mutants exhibited a similar phenotype in response to targeting dNTP pools. One explanation is that exonuclease deficient POLD1 may lead to replication fork stalling and replication stress under conditions of low dNTPs, just as proposed for the polymerase mutant. If so, the dual role of gemcitabine as an RNR inhibitor and chain terminator (Rizzuto *et al.*, 2017) may amplify induction of DNA replication stress beyond just RNR inhibition alone.

We found it surprising that POLD1 lines that only express the S605del allele are viable. Endogenous WT POLD1 may be expressed below the level of detection. Intriguingly, it has been reported that POLD1 knockout embryos (*Pold1*^{−/−}) are viable until E4.5–E7.5 and cells isolated from the *Pold1*^{−/−} blastocyst can be cultured, although with proliferation defects and increased apoptosis (Uchimura *et al.*, 2009). It should be pointed out that we utilized hTERT immortalized lines. Introduction of hTERT can confer unlimited replicative capacity and the process of hTERT immortalization may mask some of the cellular disease phenotypes, as seen with other genomic instability/progeroid syndromes, such as Hutchinson–Gilford progeroid progeria syndrome and Werner syndrome (Hisama *et al.*, 2000; Li *et al.*, 2019).

SAMHD1 is a dNTP triphosphohydrolase that is known to decrease nuclear dNTP concentration (Lahouassa *et al.*, 2012). Biological significance of SMAHD1 includes the restriction of HIV-1 (human immunodeficiency virus -1) and in the suppression of autoimmune responses (Mereby *et al.*, 2018). It is not surprising that SAMHD1siRNA improved

cell viability in the presence of HU. Unlike RRM1 and RRM2 siRNA, however, we did not observe a quantitative difference in the SAMHD1 siRNA effect among wild-type and POLD1 mutant lines during siRNA screening. However, any improvement in growth of POLD1-S605del cells could potentially translate to patients, if the fundamental cause of disease morbidity is one of impaired cell proliferation.

In conclusion, our data are consistent with the notion that hTERT fibroblasts carrying either exonuclease or polymerase mutant POLD1 are hypersensitive to inhibition of dNTP synthesis, laying the groundwork for future studies in primary fibroblasts.

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No competing financial interests exist.

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