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The enhancement of CRISPR/Cas9 gene editing using metformin

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ABSTRACT

The CRISPR/Cas9 technology is a revolutionary tool that can be used to edit the genome. Specifically, the genome of hematopoietic stem cells (HSCs) could be edited to correct monogenic blood disorders as well as produce immunotherapies. However, the efficiency of editing HSCs remains low. To overcome this hurdle, we set out to investigate the use of metformin, an FDA-approved drug, to enhance gene modification. We assessed the effect of metformin on the growth of two hematopoietic cell lines: a myeloid-erythroid leukemic cell line (K562 cells) representative of the myeloid population and an immortalized T lymphocyte cell line (Jurkat cells) representative of the lymphoid population. No significant difference in growth patterns was observed in concentrations up to 10 mM metformin in both cell lines. We then assessed the ability of two different concentrations of metformin (0.001 mM or 1 mM), based on our observations, to enhance both (1) the cutting efficiency of Cas9 and (2) the targeting efficiency with the use of a donor DNA repair template. The cutting efficiency of Cas9 was significantly enhanced in a total of five guide RNAs (four specific to a platelet locus and one specific to an erythroid locus) following treatment. In addition, an enhancement in targeting was observed with the use of a GFP-containing donor DNA repair template with both concentrations. Overall, a greater than two-fold increase in GFP expression was noted in cells treated with metformin. This suggests that metformin, an FDA-approved drug, could be added to existing protocols to enhance CRISPR/Cas9 gene editing.

1. Introduction

The CRISPR/Cas system is used as a tool to facilitate gene editing by producing directed double-stranded breaks (DSBs) in the genome. Once the DSB is produced, two main endogenous repair pathways are utilized: homology-directed repair (HDR) or nonhomologous end joining (NHEJ). When HDR occurs in cells it can utilize the sister chromatid as a DNA template to repair the DSB or an exogenous donor template can be generated and used as the DNA repair template [1]. For gene editing purposes, a donor DNA repair template can be strategically designed to contain an exogenous gene that can be inserted at the site of repair. NHEJ, on the other hand, involves the ligation of the two ends produced by the DSBs and results in insertions and deletions (indels) at the site of the break [1]. Without the need for a repair template, the NHEJ pathway is more efficient than HDR and occurs more frequently in human cells. Specifically, NHEJ occurs throughout the entirety of the cell cycle while HDR only occurs in the S/G2 phase [2].

Although CRISPR/Cas9 is a powerful tool that has been shown to be successful in gene editing of monogenic diseases, the efficiency of editing in HSCs remains low [2–5]. Editing of hematopoietic stem cells (HSCs) in particular would be useful to correct mutations found in hematological genetic diseases due to their self-renewing and differentiating nature [6]. A variety of small molecules have been postulated to enhance the modification efficiency of cells by targeting different molecular mechanisms. For example, due to competition between the two endogenous repair pathways, HDR must be favored in order to produce successful insertion of a functional transgene. L7555, resveratrol, and RS1 have been shown to promote HDR [7,8], while SCR7, NU7441, and KU-0060648 suppress NHEJ in primary cell lines [9–12]. Alternatively, synchronizing the cell cycle in the S/G2 phase with the use of nocodazole, ABT-751, and RO-3306 can facilitate the promotion of HDR as HDR solely occurs during this phase [2,13,14]. While these strategies have been shown to enhance CRISPR/Cas9 editing efficiency, it is important to note that these pathways are vital for genome stability. Thus,

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alternative mechanisms should be explored.

To enhance the editing of HSCs, we will be focusing on the small molecule, metformin. Metformin is a drug that is used to treat diabetic patients [15]. The mechanism by which metformin might help aid gene modification is unknown. However, it has been used in prior studies in conjunction with histone deacetylase inhibitors (HDACi) in which the efficacy of HDACi was increased synergistically [16]. Since the utilization of HDACi has been suggested as a potential molecule to enhance HSC modification, we, therefore, investigated the inclusion of metformin in CRISPR/Cas9 gene editing protocols.

2. Materials and methods

2.1. Chemicals and reagents

Twenty-five grams of metformin hydrochloride salt (ThermoFisher #M200925G) was dissolved in 500 mL of cell culture water (Lonza #17-724Q) to yield a final concentration of 0.3 M. Aliquots of the 0.3 M metformin solution were stored at $-20^{o}C$. Subsequent dilutions were made using the same original solvent, the cell culture water.

2.2. Plasmids

The pX330 Cas9 and guide RNA expression plasmid was acquired from Addgene [17]. Five different guide sequences specific for the *PF4* locus and four different guide sequences specific for the *RhD* locus were synthesized by Integrated DNA Technologies (Newark, NJ). These oligos were annealed in a thermocycler and phosphorylated using PNK (New England Biolabs #M0201L). A single guide RNA sequence specific for the *PF4* locus was incorporated into pX330 downstream of the U6 promoter via *Bbs*I digestion (New England Biolabs #R0539L) using T4 DNA Ligase (New England Biolabs #M0202L). A total of five PF4 and four RhD guide RNA expression pX330 plasmids were produced. Guide integration was confirmed via sequencing. The pMAX plasmid for GFP expression was used as a positive nucleofection control and acquired from Lonza.

An RhD-specific donor DNA repair template was designed to include a proof-of-principle exogenous green fluorescent protein (GFP) transgene, a bovine growth hormone polyA sequence, and two arms homologous to the target site of the human *RhD* locus. Each component was ligated into a plasmid vector from the pCR™-Blunt II-TOPO® kit (Invitrogen, Carlsbad CA) sequentially and the plasmid was then chemically transformed into DH5α *E. coli* cells (ThermoFisher #18265017). The plasmids were isolated via the endotoxin-free plasmid Maxi kit (QIAGEN #12362) and stored at room temperature.

2.3. Cell culture

Human myeloid erythroid leukemia K562 cells (ATCC #CCL-243) and human T lymphocyte Jurkat cells (ATCC #TIB-152) were cultured in 0.22 μM filtered RPMI 1640 (ThermoFisher #21870076) supplemented with 10% FBS (Sigma Aldrich #F2442-500 ML) and 1% Penicillin/Streptomycin (ThermoFisher #15140163). Cells were maintained in a 37 °C incubator in a 5% CO_2 atmosphere. Prior to experimentation, cells were kept at a concentration of at least 300,000 cells/ml to a maximum threshold of 1×10^6 cells/ml.

For growth curve analysis, cells were treated in media with a given concentration of metformin and incubated at 37 °C. 24 h after treatment, the drug was removed and the cells were washed with phosphate-buffered saline (PBS) to ensure removal of residual drug. Cells were then plated at a concentration of 300,000 cells/mL and counted every 24 h. It is important to note that no additional media was added following the initial plating.

2.4. Nucleofection

The CRISPR/Cas9 components were delivered to the nucleus of K562 and Jurkat cells using either the Lonza Nucleofector 2 b device (#AAF-1002 B) or the Lonza Amaxa 4D (#AAB-1001). Two million K562 cells were nucleofected for each condition. All conditions were performed in triplicate. The cells were resuspended in 100 μL of nucleofector solution (Lonza #VCA-1003) and the appropriate amount of plasmid DNA was added subsequently. For assessment of the cutting efficiency, 1 μg of the Cas9/sgRNA expression plasmid was used. In order to gene edit cells, 4 μg of the Cas9/sgRNA expression plasmid and 4 μg of the donor DNA repair template were used. The solution was transferred to a Lonza Human Stem Cell Nucleofector cuvette with a 0.4 cm gap as recommended for mammalian cells. The T-016 program for K562 cells was selected on the Lonza Nucleofector. Immediately following nucleofection, 500 μL of supplemented media (RPMI 1640 from ThermoFisher) was added to each sample, mixed, and transferred to their respective tissue culture wells for a three-day incubation. On the third day, the samples were analyzed. Of note, the following three standard controls were used for each set of experiments: (1) no nucleofection/no DNA (negative control), (2) nucleofection/no DNA (mock transfection control), and (3) pMAX (positive control).

2.5. DNA extraction and amplification

Genomic DNA was extracted from treated and untreated cells using the Qiagen DNeasy® Blood & Tissue Kit (Qiagen #69506). PCR amplification of the region surrounding the intended DSB was performed using the following primers:

PF4 forward primer 5′ AGCTGAAGCTGAAGAAGATGGG 3’
PF4 reverse primer 5′AACCAGTATTCACACCTTCCTTCA 3′
RhD forward primer 5′ AACTGAGCACAGCAGGAA 3’
RhD reverse primer 5′GCCCAGGCTGCTTCTAAAGG 3′

To determine if the *RhD* locus was edited to express GFP, the following primers were used. Of note, the forward primer was designed to anneal outside of the 5’ arm of homology included in the RhD-specific DNA repair template. The reverse primer was designed to anneal within the integrated GFP sequence. Thus, a PCR product could only be produced in the event of gene targeting.

Targeting forward primer 5′ TCACCCTAAGGCTGGATCAGG 3’
Targeting reverse primer 5′ ATGTTGCCGTCCTCCTTGAAGTCG 3’

For further confirmation, 5 μL of each PCR product was run on a 1% Agarose gel. The remaining PCR product underwent PCR cleanup and sequencing by McLab (South San Francisco, CA). An alignment was performed to verify integration of our donor DNA repair template to the targeted genomic location.

2.6. Measurement of allele modification using TIDE

250 ng of genomic DNA of each sample was used to PCR amplify the region of interest. The purified products were sequenced using McLab Sanger-sequencing, which provided chromatograms of the individual samples, including an untreated sample as a negative control. These chromatograms were then input into an algorithm-based online TIDE (Tracking of Indels by Decomposition) software (http://tide.nki.nl). This software estimates the allele frequency of insertions and deletions within the sequence as compared to the negative control as previously described [18]. Parameters were adjusted to ensure that the decomposition window included the greatest possible amount of high-quality trace sequence. The maximum indel size was set at ten nucleotides.

2.7. Statistical analysis

The GraphPad Prism software was used to compile and analyze the data. For comparing the means of two separate data groups, an unpaired Student *t*-test was utilized. Otherwise, for the comparison of three

groups, a one-way Analysis of Variance (ANOVA) was used to assess significance. P values less than 0.05 were considered to be statistically significant. Figures were produced and exported such that the results are displayed as the mean ± standard deviation.

3. Results

3.1. Metformin does not affect the growth potential of hematopoietic cell lines

The effect of metformin on the growth curve of two hematopoietic cell lines was evaluated. The myeloid erythroid leukemic K562 cell line and the immortalized T lymphocyte Jurkat cell line were selected as representative of the two lineages derived from the HSC. Both cell lines were treated with concentrations of metformin ranging from 100 mM to 0.0001 mM (Fig. 1A). No fresh media was added after initial washing to ensure that any effect on cellular proliferation was being adequately evaluated as a consequence of metformin. Thus, cellular proliferation was expected to cease as was observed in the untreated cells. No difference in growth pattern was observed between the untreated K562 cells and the 10 mM ($p = 0.7047$), 1 mM ($p = 0.6150$), 0.1 mM ($p = 0.4353$), 0.01 mM ($p = 0.3954$), 0.001 mM ($p = 0.3941$), and 0.0001 mM treated cells ($p = 0.1483$) (Fig. 1B). A significant difference, however, was observed in the growth of K562 treated with 100 mM metformin ($p = 0.0013$). Of note, viability was not decreased in cells treated with both a higher concentration of metformin (1 mM) and a lower concentration of metformin (0.001 mM) (Supplemental Fig. 1). Instead, a significant increase in viability was observed. Similarly, no difference was observed between the untreated Jurkat cells and the 10 mM ($p = 0.5782$), 1 mM ($p = 0.2768$), 0.1 mM ($p = 0.8959$), 0.01 mM ($p = 0.5116$), 0.001 mM ($p = 0.6056$), and 0.0001 mM ($p = 0.9363$) (Fig. 1C). A significant difference was also observed in the growth of Jurkat cells treated with 100 mM metformin ($p < 0.0001$). Taken together, the metformin concentrations of 1 mM and 0.001 mM were chosen as optimal concentrations and used for the nucleofection experiments.

3.2. Metformin enhances the cutting efficiency of a set of CRISPR guide RNAs for the purpose of platelet-specific expression

Platelet factor 4 (PF4) is a cytokine released from the α-granules of platelets following platelet activation [19]. PF4 assists in inhibiting antithrombin, and as such is considered pro-coagulatory. A set of five different guide RNAs specific to the *PF4* locus were produced and assessed for cutting efficiency in K562 cells (Fig. 2A). Of note, the *PF4* locus is not found to be expressed in K562 cells (proteinatlas.org) [20,

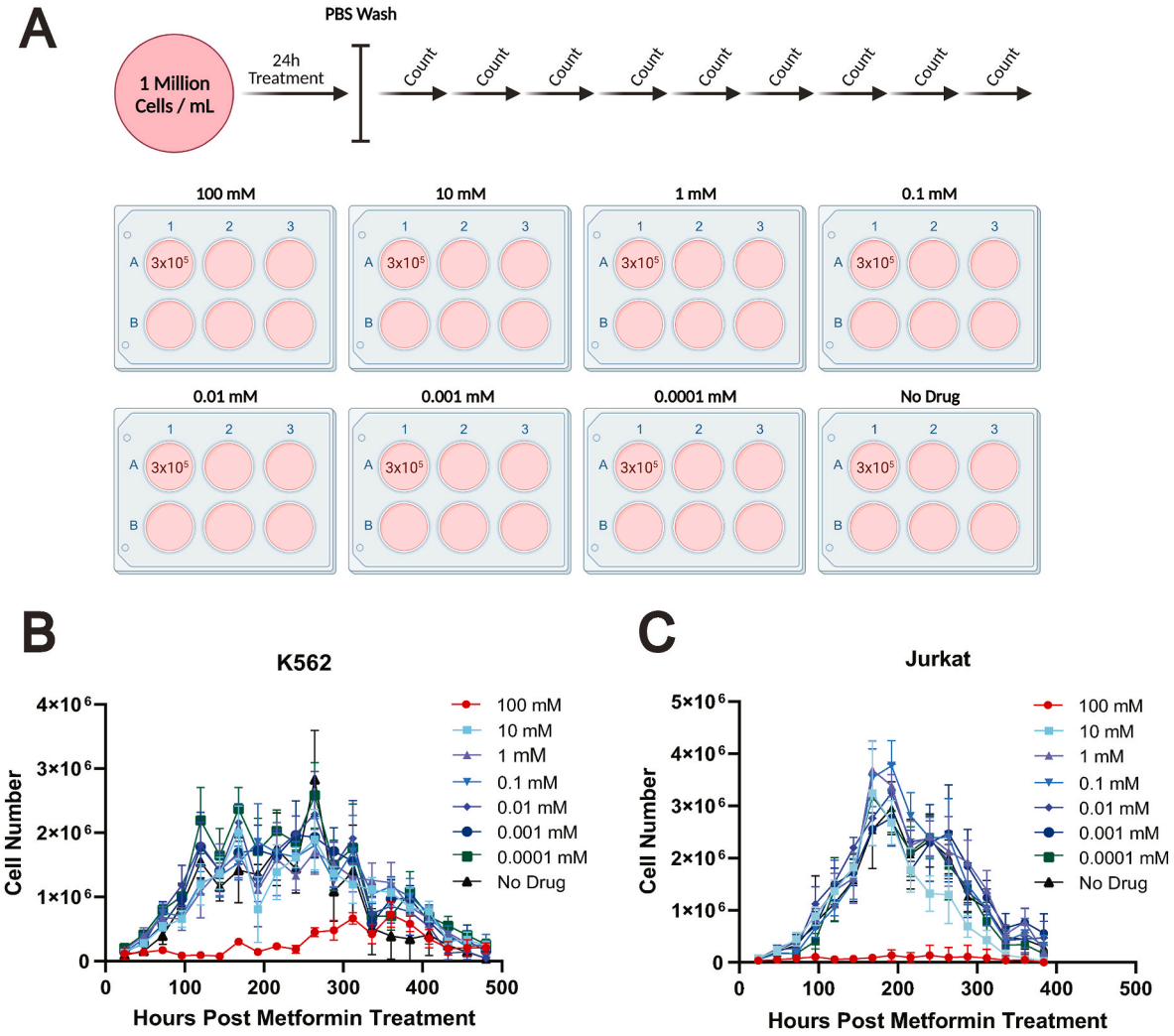

Fig. 1. Effect of metformin on growth curves of K562 and Jurkat cells.
A) Schematic representation of the experimental design. Cells were treated with metformin for 24 h and then replated to be counted every following 24 h. Notably, no additional media was added after initial washing. B) K562 cells were counted up to twenty days post-drug removal. All concentrations were performed with an n = 6. C) Jurkat cells were counted up to 16 days following drug removal. All concentrations were performed with an n = 6.

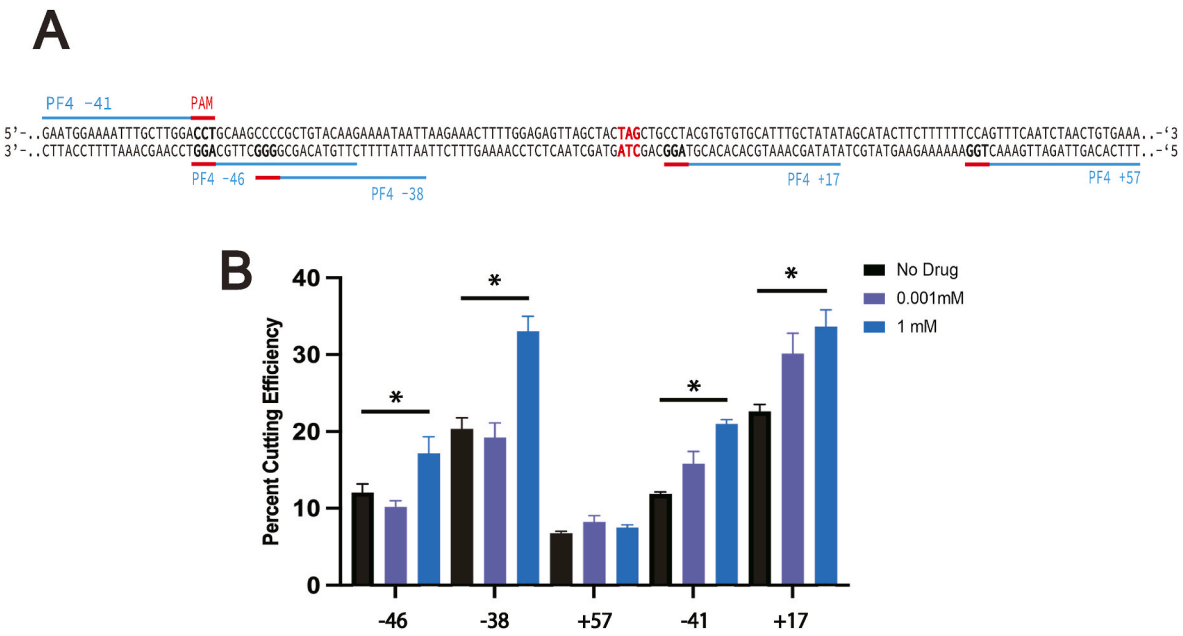

Fig. 2. Effect of metformin on cutting efficiency of *PF4* CRISPR guide RNAs.
A) Schematic layout of guide RNAs specific to the *PF4* locus designed for this experiment. Red lines indicate the protospacer adjacent motif (PAM) sequences. B) K562 cells were treated with 0.001 mM and 1 mM of metformin and nucleofected with one of five PF4 CRISPR guide RNAs. TIDE analysis was performed on amplified genomic DNA for indel frequency. The figure represents the mean of independent treatment groups of each guide. Statistical significance was quantified using a one-way ANOVAand p-values <0.05 are denoted with an *. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

21]. The cells were nucleofected with a Cas9-expressing plasmid containing each PF4 guide RNA 24 h after pretreatment with metformin. After a 72-h incubation period, the genomic DNA was isolated and PCR amplified for analysis of the cleavage site. Using the online TIDE software [17], the frequency of indels was quantified to ascertain each guide RNA's cutting efficiency. An enhancement in all but one guide RNA was noted following pretreatment with metformin for 24 h. Comparison between the three groups yielded a statistically significant difference with the −46 ($p = 0.0365$), −38 ($p = 0.0026$), −41 ($p = 0.002$), and +17 ($p = 0.0185$) PF4 guides. Notably, compared to the untreated group, the cutting efficiency of the +17 guide was enhanced with both 0.001 mM metformin ($p = 0.0467$) and 1 mM metformin ($p = 0.0098$) (Fig. 2B) (Supplementary Fig. 2). However, the greatest enhancement was noted with the higher concentration of metformin in that the cutting efficiency

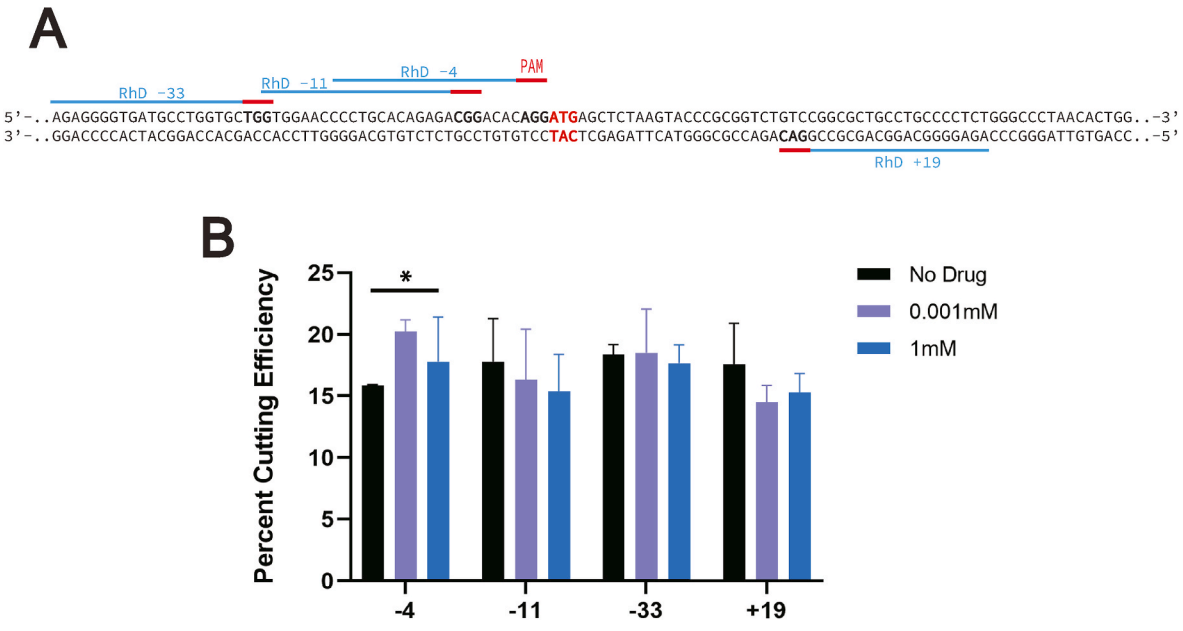

Fig. 3. Effect of metformin on cutting efficiency of RhD CRISPR guide RNAs.
A) Schematic layout of guide RNAs specific to the *RhD* locus designed for this experiment. B) K562 cells were treated with 0.001 mM and 1 mM of metformin and nucleofected with one of the four RhD CRISPR guide RNAs. TIDE analysis was performed on amplified genomic DNA for indel frequency. The figure represents the mean of independent treatment groups of each guide. Statistical significance between untreated and treated groups was evaluated using a one-way ANOVA and p-values <0.05 are denoted with an *.

of the −41 guide (p = 0.0002) and the −38 guide (p = 0.0063) was increased (Fig. 2B). Although not significant, a similar trend was noted with the −41 guide treated with 0.001 mM (p = 0.0726) (Fig. 2B). Overall, a significant enhancement in cutting efficiency was observed following pretreatment with metformin.

3.3. Metformin minimally enhances the ability of an erythroid-specific CRISPR guide RNA to produce a double-strand break

The *RhD* (Rh blood group D antigen) gene encodes for RhD proteins on erythrocyte membrane surfaces, which results in the classification of a positive blood type. When this gene is absent or deficient, an individual is denoted as having an Rh-negative blood type. In contrast to the *PF4* locus, the *RhD* locus has been noted to be minimally expressed in K562 cells (proteinatlas.org) [20,21]. A set of guide RNAs homologous to the *RhD* locus (Fig. 3A) were assessed for effects in cutting efficiency in K562 cells pretreated with metformin. Following the aforementioned experimental procedure as performed with the PF4 guide RNAs (Fig. 2B), K562 cells were treated with 0.001 mM or 1 mM of metformin and nucleofected with an RhD guide RNA within a Cas9 expressing plasmid. A statistically significant enhancement in cutting efficiency of one guide RNA (−4 RhD) was noted overall (p = 0.0246). Specifically, compared to K562 cells that were not treated prior to nucleofection, the concentration of 0.001 mM induced a statistically significant enhancement in cutting efficiency with the −4 RhD guide RNA (p = 0.0374), however, 1 mM did not have a significant change (p = 0.7043) (Supplemental Fig. 3). Metformin did not produce a significant difference in the cutting efficiencies of the −11 (p = 0.5008), −33 (p = 0.9621), and +19 (p = 0.6185) RhD guide RNAs (Fig. 3B).

3.4. Metformin enhances editing efficiency of the CRISPR/Cas9 system

The effectiveness of metformin in enhancing CRISPR/Cas9 editing efficiency was then assessed in K562 cells. Using the same experimental procedure as done for evaluating CRISPR guide RNA cutting efficiency, K562 cells were pretreated with either 0.001 mM or 1 mM of metformin. Following drug removal, treated and untreated cells were nucleofected with the −4 RhD guide RNA and the RhD-specific donor DNA repair template (Fig. 4A). Additionally, three untreated groups of cells were nucleofected with either pMAX serving as a positive control or the −4 RhD guide RNA expressing plasmid alone or no plasmid DNA as negative controls. GFP expression was quantified 72 h after nucleofection via flow cytometry (Fig. 4B). Though both untreated and treated cells produced GFP expression (Fig. 4C–E), there was a statistically significant 2.9-fold and 2.5-fold increase of expression in cells treated with 0.001 mM and 1 mM metformin respectively (p = 0.001) (Fig. 4F). Through subsequent sequencing, the GFP expression was confirmed to originate from the *RhD* locus as a result of targeted integration (Supplemental Fig. 4).

4. Discussion

Metformin was approved by the FDA in 1994 and has since become the most widely prescribed oral anti-diabetic drug [22]. Metformin, a biguanide, is a synthetic derivative of galegine which was originally isolated from the French lilac *Galega officinalis* [23]. As a type II diabetes therapeutic, metformin physiologically reduces blood glucose levels by decreasing hepatic gluconeogenesis [24,25]. However, the underlying molecular mechanism by which these beneficial effects occur has yet to be fully elucidated and is likely to be due to a variety of molecular interactions [26].

One of these interactions involves adenosine monophosphate-activated protein (AMP)-activated protein kinase (AMPK). Metformin has been observed to enhance the activity of AMPK as a result of direct inhibition of mitochondrial complex I [27,28]. AMPK is involved in maintaining energy homeostasis by regulating cellular ATP reserves. Specifically, AMPK phosphorylates acetyl-CoA carboxylase (ACC). Phosphorylated ACC is unable to convert acetyl-CoA to malonyl-CoA, a rate-limiting step involved in the synthesis of fatty acids. This in turn increases cellular acetyl-CoA levels. An increase in acetyl-CoA is associated with an increase in histone acetylation [29]. Histone acetylation converts the chromatin configuration from a closed conformation to an open conformation. A more open chromatin configuration has been suggested as a mechanism to enhance genome editing by increasing the accessibility of the CRISPR/Cas9 machinery [30,31]. Recently, we noted the ability of valproic acid and sodium butyrate to increase CRISPR/-Cas9 editing efficiency based on their ability to inhibit histone deacetylation (HDAC) [32]. Metformin has been used in prior non-genome editing studies in conjunction with the class I/II histone deacetylase inhibitor (HDACi) trichostatin A in which the efficacy of the HDACi was increased synergistically [16]. Since the utilization of HDACi has been suggested as a potential molecule to enhance HSC modification, we, therefore, investigated the inclusion of metformin in CRISPR/Cas9 gene editing protocols.

Several concentrations of metformin were observed to be well tolerated in two hematopoietic cell lines representative of the two lineages derived from HSCs. Of note, concentrations as high as 10 mM did not yield an observable effect on cellular proliferation profiles. In addition, no morphological change in either K562 or Jurkat cells was noted (data not shown). Given this, we choose to utilize two concentrations of metformin, a high (1 mM) and a low conservative concentration dose (0.001 mM) in our subsequent assessment of the ability of metformin to enhance genome accessibility of the CRISPR/Cas9 system.

An enhancement in the cutting efficiency of several CRISPR guide RNAs was noted following the implementation of metformin. Specifically, four of the five PF4 guide RNAs (−46, −38, −41, and +17) underwent heightened cutting efficiency following pretreatment of 1 mM metformin (Fig. 2B). When assessing a second set of CRISPR guide RNAs specific to the *RhD* locus, the effect was not as evident. Only one guide RNA, (−4 RhD guide RNA) was enhanced following pretreatment of 0.001 mM metformin (Fig. 3B). Interestingly, no statistically significant enhancement was observed with the higher concentration of metformin. This suggests that the effect of metformin on CRISPR/Cas9 cutting efficiency may be locus-specific or less effective at a locus that is already more readily accessible as is the case with the *RhD* locus compared to the *PF4* locus.

Being that an enhancement in cutting efficiency was observed with the −4 RhD guide RNA, this guide was used to assess if HDR efficiency could also be enhanced via the implementation of metformin. Following the production of a double-strand break, a donor DNA repair template containing a fluorescent transgene could be used to repair the break via the endogenous HDR pathway and readily observe gene targeting. The amount of successfully edited GFP-positive cells was significantly increased by 2.9-fold and 2.5-fold following pretreatment of 0.001 mM and 1 mM of metformin respectively. The enhancement from metformin that we observed is likely due to increased accessibility of CRISPR/Cas9 and the gene editing donor DNA repair template to an even more openly configured genomic region as is required for HDR to take place.

Together, these experiments demonstrate that metformin can be utilized to provide significant enhancement in both CRISPR/Cas9 cutting and editing efficiencies. The precise mechanism by which it does so remains to be confirmed, yet it is likely that metformin contributes to a histone conformation that is more accessible for genome modification. Further analysis is needed to confirm if a similar synergistic enhancement in gene editing could be observed with an HDACi as previously noted [17]. However, overall, our study suggests that the concentrations of metformin as low as 0.001 mM can be used to improve CRISPR/Cas9 gene editing protocols, and may be a promising contribution to future clinical applications.

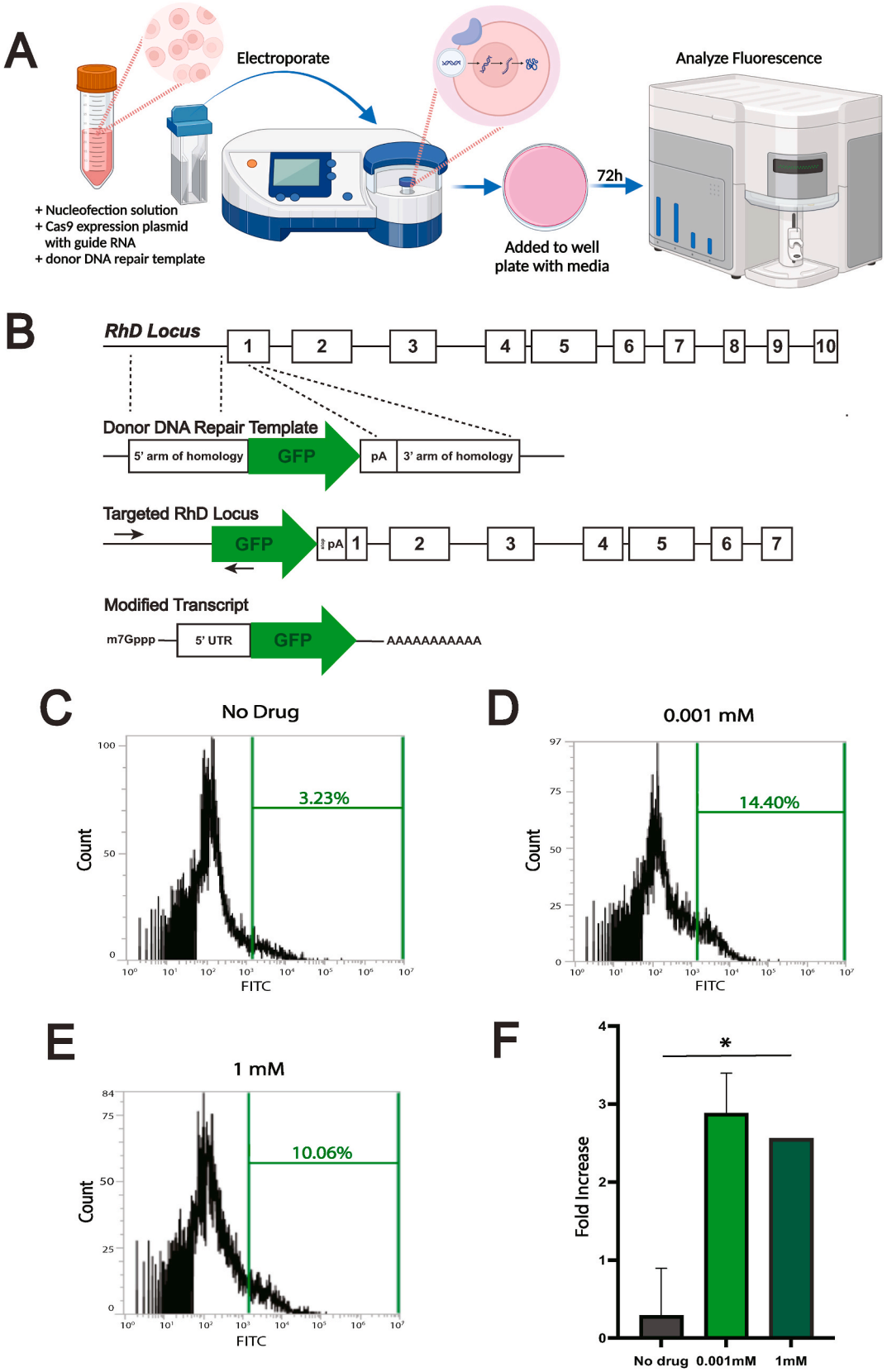

Fig. 4. Effect of metformin on CRISPR/Cas9 targeting efficiency.
A) Schematic depicting experimental design. B) Representation of targeting strategy. Primers used for subsequent analysis are indicated by small black arrows. *C*-E) GFP expression was quantified by flow cytometry. Output of each sample is displayed with the percentage of fluorescence: C) untreated cells, D) cells treated with 0.001 mM metformin, and E) cells treated with 1 mM metformin. F) Quantification of GFP expression was assessed for statistical differences. Statistical significance is denoted here as having $p < 0.05$.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jennifer Johnston reports financial support was provided by National Institute of General Medical Sciences. Jennifer Johnston reports financial support was provided by California State University Program for Education and Research in Biotechnology.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101539.

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