Effective downregulation of BCR-ABL tumorigenicity by RNA targeted CRISPR-*Cas13a*

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Abbreviations: CML, Chronic myeloid leukaemia**; CRISPR,** Clustered Regularly Interspaced Short Palindromic Repeats; **crRNA**, CRISPR RNA

Abstract

CML is a clonal myeloproliferative disorder of pluripotent stem cells driven by a reciprocal translocation between chromosome 9 and 22, forming a BCR-ABL fusion gene. Tyrosine-kinase inhibitor drugs like imatinib are the mainstay of treatment and cases resistant to these drugs have a poor prognosis in the absence of a compatible stem-cell donor. However, with rapid advancements in gene-editing technologies, most studies are now focusing on developing a translational model targeting single-gene disorders with a prospective permanent cure. The present study explores the potential application of the RNA targeting CRISPR-*Cas13a* system for effective knockdown of *BCR-ABL* fusion transcript in a CML cell line, K562. The CRISPR system was successfully able to lower the expression of downstream genes (*pCRKL* and *pCRK*) dependent on the activated *BCR-ABL* kinase signal by up-to 4.3 folds. The viability of the CRISPR treated cells were also significantly lowered by 373.83-fold (p-value= 0.000891196). The time-dependent kinetics also highlighted the significant in-vitro suppressive activity to last up to 8 weeks (p-value: 0.025). As per the cDNA sequencing data from Oxford MinION next-generation sequencer, the CRISPR treated cells show 62.37% suspected cleaved reads. These preliminary results highlight an excellent potential application of RNA targeting CRISPRs in Haematological neoplasms like CML and should pave way for further research in this direction.

1. Introduction

Chronic myeloid leukaemia (CML) is caused by a reciprocal translocation between chromosome 22 and 9, resulting in a chimeric *BCR-ABL* protein having kinase activity [1]. The translocation is characteristically between band q34 of chromosome 9 bearing Abelson (*ABL*) proto-oncogene and band q11 of chromosome 22 bearing the breakpoint cluster region (*BCR*) gene [2]. The *BCR-ABL* fusion gene protein can vary from 190 kDa to 230 kDa depending upon the breakpoint site on *BCR* gene [3]. Most commonly found *BCR-ABL* fusions carry their breakpoints in the 2.9 kb region between exons 13 and 15 (b2 and b4) of *BCR* region that fuses with the large intronic region of *ABL* between exons 1b and 2b. This forms an 8.5 kb *BCR-ABL* transcript containing *BCR* exon b2/b3 and *ABL* exon 2 (a2). Both combinations (b1a2 and b3a2) code for the same 210 kDa protein [3]. However, irrespective of the breakpoint, the *BCR-ABL* chimeric kinase phosphorylates and activates downstream proteins like *pCRKL*, *pCRK*, *pSTAT5* etc, that further modulate expression of target genes to give proliferative advantage to the Philadelphia chromosome (Ph) carrying pluripotent stem cells [4].

With recent advancements in gene editing research, much focus has drifted towards targeting single gene disorders for possible therapeutic cure. In this regard, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), has emerged as a highly versatile, flexible and cost-effective tool for gene editing experiments. This tool has circumvented the shortcomings of the previous tools like transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) with improved overall turnaround time [5-7]. In haematological neoplasms, the chimeric fusion kinase *BCR-ABL* of CML, seems an exciting target for CRISPR as it is the driving force behind the neoplastic pathogenesis. Working on the same idea, in 2017, García-Tuñón *et al*, demonstrated the use of CRISPR *Cas9* for targeting *BCR-ABL* chimeric trans-gene [8] in a synthetically engineered Boff-p210 cell line bearing the transgene coding for the p210 variant of the *BCR-ABL* fusion gene. This extensive work showcased for the first time the application of CRISPR in down regulating the *BCR-ABL* chimeric gene. However, in actual cases of CML, the breakpoints might differ at the DNA level due to different intronic breakpoint regions in ABL gene [9]. Hence, using DNA targeting CRISPR

will require a per case targeted sequencing and custom CRISPR sgRNA/ sgRNAs to effectively knockdown the *BCR-ABL* chimeric gene. This would require a dedicated research team which will eventually incur substantial costs and increase the turnaround time per case. Also, the custom target effectiveness might vary over the range of patients and hence a definitive prognosis might never be established. All these issues can be circumvented by employing techniques that can target the *BCR-ABL* transcript rather than the DNA as the former will remain immune to the intronic changes at the DNA level. This was only possible after the recent development and assessment of RNA targeting CRISPRs [10]. These CRISPR systems, unlike DNA targeting *Cas9*, are additionally more flexible and exhibit less off-target and more on-target activities. In the current experimental study, we've explored the use of CRISPR *Cas13a*, a form of RNA targeting CRISPR, in knocking down the *BCR-ABL* transcript and quantified the downstream gene products (*pCrKL* and *pCrK*) using flow-cytometry and western blot techniques, resulting in a significantly large reduction in the viability of the treated K562 cells. The overall activity of CRISPR has been enumerated by deep sequencing of cDNA by Oxford Nanopore Technologies MinION system. The developed CRISPR system was found to be effectively down regulating the levels of downstream genes of the CML pathogenesis pathway. Moreover, we have tried highlighting the time dependent kinetics of downstream effects of the editing. To the best of our knowledge, this is the first such *in vitro* experimental study successfully employing RNA targeting CRISPR-*Cas13a* in knocking down the *BCR-ABL* transcript and is likely to pave way for further research in the field.

2. Materials and Methods

2.1 Construction CRISPR plasmids

The CRISPR RNA (crRNA) was designed using CRISPR-RT server [11]. Only the crRNA designs overlapping *BCR* and *ABL* breakpoint region without any predicted off targets were considered. The top and bottom crRNA oligos with *BbsI* overhangs were procured from Sigma-Aldrich (St. Louis, Missouri, US). The two oligos were phosphorylated using T4 polynucleotide kinase and ligated using T7 polymerase to form a double stranded crRNA as per the protocol described by Cong *et al,* 2014 [12]. The plasmids *pC016 - LwCas13a guide expression backbone with U6 promoter* (Addgene #91906) and *pC034 - LwCas13a-msfGFP-2A-Blast* (Addgene

#91924) were obtained as gifts from Dr Feng Zhang. The plasmid pC034 carries blasticidin resistance and green fluorescent protein (GFP) expression markers. The plasmid pC016, was digested using *BbsI-HF* (New England Biolabs, MA, USA, Cat#R3539L) as per the manufacturer's protocol with slight modification of over-digesting the plasmid in 10U of the enzyme thrice in continuity with 16hrs of incubation each time without deactivating the enzyme in between ensuring a successful second cut after the plasmid is linearized by the first cut. The pC016 plasmid with insert (pC016I) was then transformed into competent *Escherichia coli DH5*α cells using heat shock method as described by Sambrook and Russell [13]. Plasmid was extracted from the positive clone using QIAprep Spin Miniprep kit as per the manufacturer's protocol.

2.2 Transfection of K562 cells with CRISPR plasmids

Lipofectamine LTX (cat#15338100, Thermo-Fisher Scientific, MA, US) was used for the co-transfection of the two plasmids, pC016I and pC034 using the manufacturer's protocol in a six well plate containing approximately 50,00,00 cells from overnight culture of K562 cells in RPMI-1640 medium with 1% Penicillin-Streptomycin at a confluency of 75-80%. The cells were incubated overnight at 37°C with 5% CO_2. After the overnight incubation, old media was replaced with fresh and additionally 6 µg/mL of blasticidin antibiotic was added as the positive selection marker for the plasmid in both test and negative control cells [14]. Cultures were observed till all negative control cells were killed and GFP expression of the transfected cells became close to hundred percent. Cells were harvested at passage number eight, seventeen and thirty-six for downstream analysis and time dependent kinetics study.

2.3 Downstream gene regulation analysis

Total RNA was extracted using RNeasy kit from QIAGEN (cat#74104) as per the manufacturer's protocol. A fraction of RNA was reverse transcribed to cDNA using RevertAid first strand cDNA synthesis kit by Thermo Fisher scientific (cat#K1622) using the manufacturer's protocol. The *BCR-ABL* cDNA region was amplified using specific primers, forward 5'-ACAGAATTCCGCTGACCATCAATAAG-3', reverse 5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3' [15]. For the flow cytometric analysis of *pCRKL* levels,

the *pCRKL (Y207)* specific Alexa Fluor 647 tagged antibody (cat#560790, Becton, Dickinson and Company, NJ, US) was used as per the manufacturer's protocol in a Beckman Coulter Gallios flow cytometer (Beckman Coulter Inc., IN, US). The fold change was calculated by comparing the mean fluorescence intensity (MFI) using median population and at least 10,000 events per test between K562 cells as control and K562 cells bearing CRISPR plasmids (coded as K562-1634). The calculations were done in Kaluza Analysis software by Beckman Coulter.

Whole cell protein extraction for western blot was done using Radioimmunoprecipitation assay (RIPA) buffer (cat#89900, Thermo Fisher Scientific, MA, US) using the manufacturer's protocol. Bicinchoninic acid (BCA) protein assay was done for extracted protein quantification using Pierce BCA Protein assay kit according to the manufacturer's protocol (cat#23225, Thermo Fisher Scientific, MA, US). For western blotting, primary rabbit raised antibodies against *pCRKL Y207* (37 kDa) and *pCRK Y221* (34 kDa) (another downstream target protein) were procured from Abcam plc, Cambridge, UK with catalogue numbers ab52908 and ab76227 respectively and housekeeping mouse raised *β-tubulin* (51 kDa) primary antibody was procured from Cell Signalling Technologies, MA, US with catalogue number 2146S. The anti-rabbit and anti-mouse secondary horseradish peroxidase linked antibodies were procured from Cell Signalling Technologies with catalogue numbers 7074P2 and 7076P2 respectively. Image J software was used for analysis of the western blot imaging data [16].

2.4 Cell viability assay

The cell viability assay was performed in triplicate using Promega *CellTitre-Glo* 2.0 reagent as per the manufacturer's protocol for establishing the role of knock-down of *BCR-ABL* transcript on the cells.

2.5 Enumeration of CRISPR activity by NGS

Total RNA from K562-1634 and K562 cells were subjected to cDNA sequencing using Oxford Nanopore Technologies MinION instrument with SQK-PCS108 library kit along with barcodes from SQK-PBK004 kit on a FLO-MIN106 R9.4 flow cell according to the manufacturer's protocol. QCAT tool was used for demultiplexing the barcoded reads (https://github.com/nanoporetech/qcat), NanoFilt was used to filter the data with minimum

quality cut off of 5 [17]. NCBI local BLAST+ was used to extract raw reads carrying crRNA sequence [18]. The reads carrying the crRNA sequence were extracted from the whole reads using NCBI Basic alignment search tool (BLAST) [18] and then aligned with *BCR-ABL* cDNA and crRNA sequences using BWA-MEM[19]. The resulting SAM alignment format was coordinate sorted and indexed using SAMtools[20] and visualized in Integrative Genomics Viewer (IGV)[21]. The study was duly approved by the Institute's Ethics Committee and the Departmental Review Board.

2.6 Statistical analysis

SPSS Statistics software version 25 (IBM Corp, NY, US) was used to perform statistical tests. The flow cytometry and western blot data was normalized using maximum value before performing any statistical tests. Student's t test was used to check for significant change in expression over the period. Pearson's correlation was used to find significant relation between flow and western blot data.

2.7 Data Availability

Data sharing is not applicable to this article as no new data were created or analysed in this study.

3. Results

3.1 crRNA Designing

CRISPR-RT suggested a total of 175 target regions, out of which, 14 were overlapping the *BCR-ABL* chimeric region. Among these 14 candidate crRNAs, the one 5'-ATTTAAGCAGAGTTCAAAAGCCCTTCAG-3', which spanned significantly in both *BCR* and *ABL1* regions (17 and 12 bases in *BCR* and *ABL1* respectively) with the lowest GC content of 39% and no predicted off targets was chosen. There were no predicted off-targets because *Cas13a* tolerates only one mismatch and in case of our crRNA design, the resulting CRISPR won't be able to target wild type BCR or ABL because of 12 and 17 bases mismatch respectively.

3.2 Transfection and downstream analysis

Pure colonies of K562-1634 transfected cells were selected by day 12 (figure 1). Levels of *pCRKL* in control K562 and K562-1634 cells as assessed by flow cytometry at passages eight, seventeen and thirty-six is given in table I. Flow cytometric analysis was done in duplicate for each run and the average was used for calculations. At least ten thousand events per reaction were recorded with maximum events recorded reaching around 85,000 in a single reaction. *pCRKL* level was observed to be 4.30, 4.02 and 1.80 times lower than the control K562 cell levels at passages eight, seventeen and thirty-six respectively (p-value 0.025) (figure 2A). Western blotting data of *pCRKL* and *pCRK* (table II, figure 2 B and unedited pictures in supplementary figure S1-S3) was found to be synchronous with the flow cytometry data after normalization with a significant p-value of 0.01 (figure 2 C). Cell viability assay performed for passage 11 cells in triplicate for control and treated cells after compensating the blank readings were, 1300479, 1237852, 1173013 and 1300479, 1237852, 1173013 respectively. This translates into a 373.83-fold (p-value: 0.000891196) reduction in the viability of the cells when treated with CRISPR (figure 3).

3.3 NGS sequencing of transfected and control cell lines

The next generation sequencing using Oxford MinION generated a total of 5,365,731 reads with an average read length of 402.8 bp, median read length of 253 bp and maximum read length of 232,778 bp. As far as the calculation of the CRISPR activity, we found all the reads to

be shorter than the total reads on in the alignment data but since the NGS reads are short and incomplete, only the reads which were found to begin and end abruptly around 112 bp in the 257 bp focus region of the *BCR-ABL* transcript upstream of the crRNA region were accounted for CRISPR activity. According to this, out of the 93 reads, 58 reads were cleaved by the CRISPR activity, which translates to a 62.37% activity (supplementary figure S4). Interesting to note here was the fact that the reads also show base substitutions and INDELs in the crRNA region but *Cas13a* is not known to cause them hence we've associated them to the platform errors of the sequencer.

4. Discussion

Tyrosine kinase inhibitor (TKI) drugs, though being the mainstay of treatment in CML, have been shown in studies to have adverse effects on growth of children in addition to neoplastic clones developing resistance to their action during long term treatment [22]. In a retrospective study by Millot *et al.,* in 2014 which included 81 children under 18 years of age with CML, it was found that imatinib adversely affected growth in the subjected children [23,24]. Additionally, there are several cases where resistance against these drugs are forged, these cases then either require a complex treatment regime including trials with other TKI drugs or ultimately a stem cell transplantation (SCT) [25]. SCT has its own set of complications and issues. First, it is hard to find a compatible donor, if found, there are chances of development of graft-versus-host diseases (GVHD) [26]. Even if everything is in place, treatment costs become unaffordable for most people, especially from developing countries like India. Gene editing based corrective techniques might prove to be beneficial in such conditions in long term considering their current efficiency and flexibility. There are four major genome editing tools, namely, Meganucleases (MNs), ZFNs, TALENs and the most recent, CRISPR. CRISPR finds its edge in being almost 10 times cheaper than its closest competitor, TALENs, has widest applications, is freely available and simplest to design and apply [27]. Earlier, in a study by García-Tuñón *et al,* it was observed that CRISPR *Cas9* was able to effectively control the oncogenic capabilities of *BCR-ABL* chimeric gene in custom Boff-p210 cell line carrying *BCR-ABL* fusion transgene [8]. In a recent study, Liu *et al* demonstrated effective down regulation of *BCR-ABL* gene using CRISPR

RNA guided *FokI* Nucleases in both cell lines and murine xenograft model [28]. However, García-Tuñón *et al,* in their other research article on CRISPR, published recently, highlighted the limitation of CRISPR/Cas9 in effectively knocking out target DNA without a null variation inducing homology directed repair template in the construct. They went on to demonstrate that effective knockdown could be achieved if sgRNAs target the splice donor site of the gene [29]. Moreover, it has been shown that in CML, using a DNA targeting CRISPR would mean designing custom guide RNAs per patient as the breakpoints might differ per case at the DNA level [9]. Considering above limitations and problems in targeting *BCR-ABL* gene at DNA level by CRISPR/Cas9, we hypothesized utility of targeting the p210 transcript of CML with an RNA CRISPR since the p210 transcript wouldn't be likely affected by the differences in intronic breakpoints. Working on this hypothesis, we designed this study to explore the potential of RNA targeting CRISPR *Cas13a* in knocking down the tumorigenicity of the *BCR-ABL* chimeric gene. CRISPR *Cas13a* is a relatively newer form of CRISPR which targets RNA rather than the DNA and has been shown to have an efficiency of around 50% [10]. In the current study too, we found the efficiency to be around 57.45%. This RNA targeting CRISPR system was able to lower the expression of downstream genes of CML pathogenesis as per the KEGG [30] pathway ID hsa05220.

pCRKL levels as calculated through passage numbers 8, 17 and 36 were 23.26, 24.85 and 55.43% respectively by flow cytometric analysis and 15.75, 17.52 and 54.00% respectively by western blotting experiment. The same for *pCRK* was found to be 37.84, 38.23 and 77.80% through passage numbers 8, 17 and 36 respectively as per the western blotting data. All the cells were killed in presence of blasticidin antibiotic by passage number 42. This tells us that the cells started to lose plasmid around passage number 36 and by 42 they had all lost it. This suggests that the RNA targeting CRISPR *Cas13a* in vitro is likely to persist till a month with no significant decrease in effectiveness. Post that time, the effectiveness starts deteriorating which gradually comes down to the cells losing the plasmid. Also, the cell viability assay confirmed that the cells were significantly less viable than the control cells. This was because the BCR-ABL gene provides a proliferation advantage to the cells and when its transcripts

were knocked down by CRISPR, it also led to the drop in the viability of the cells. On the analysis of the *BCR-ABL* transcript changes by NGS, we could assert an overall 62.37% cleavage activity of CRISPR Cas13a based on the truncated reads upstream of the crRNA region, which is a known mechanism as described by Wolter, F. et al., in 2018 [31]. Overall, our study results show that CRISPR-*Cas13a* efficiency (based on NGS) and down regulation of downstream genes (*pCRKL* & *pCRK*) go hand in hand *in vitro*. The whole study is summarised in a self-explanatory graphical summary illustrated in supplementary figure S5. The study is limited by further analysis on primary culture of multiple CML patients. Utilizing newer, more efficient RNA targeting CRISPRs like CRISPR *Cas13b* and *Cas13c* with efficiencies going up to 90-9*5%* [32] can potentially revolutionize the effects further with higher fold reduction in downstream target protein expression, ultimately reducing the tumorigenic capabilities of *BCR-ABL* chimeric gene. It is interesting to mention here that imatinib in a dose dependent manner of 10 ≥μM reduces *pCRKL* levels to 16.6% which translates to a 6.05-fold reduction [33], compared to 4.3-fold reduction achieved by the described CRISPR *Cas 13a* RNA machinery. However, as detailed above, with rapid advancements in the CRISPR technology, this gap might be filled or even surpassed in near future.

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Figure Title & or legends

Figure 1: Blasticidin selection of K562-1634 cells over different passages in contrast with wild type K562 cells**.** A1-A3 and B1-B3 Inverted microscopy images taken at 10x magnification on days 08, 10 and 12 for control K562 and 1634 plasmid bearing K562 cells respectively. C: Final resulting cell population of 1634 plasmid bearing cells at 10x magnification showing widespread GFP expression.

Figure 2: Flow cytometry and western blot data for control vs K562-1634 cells. A: Flow cytometry data for comparative *pCRKL* expression levels. P08, P17 & P36 denote passage numbers 8, 17 and 36 for K562 cells bearing CRISPR plasmids (K562-1634 cells). B: Comparative western blot data for *pCRK* and *pCRKL* expression levels. K- K562 cells without plasmids (control). P08, P17 & P36 denote passage numbers 8, 17 and 36 for K562 cells bearing CRISPR plasmids (K562-1634 cells). C: Comparative trends between western blot and flow cytometry data after normalization.

Figure 3: Cell viability comparative assay between un-transfected K562 control and CRISPR treated K562 cells (K-1634).

Supplementary Data

Supplementary Figure S1: Unedited raw western blot image for *β-tubulin* estimation

Supplementary Figure S2: Unedited raw western blot image for *pCRKL* estimation

Supplementary Figure S3: Unedited raw western blot image for *pCRK* estimation

Supplementary Figure S4: cDNA reads aligned to reference *BCR-ABL* region from the Oxford MinION sequencing data. A: Control K562 reads. B: Reads from K562 cells bearing the CRISPR plasmids showing cleaved reads.

Supplementary Figure S5: Graphical summary of the work.

Table I: Fold change of *pCRKL* assessed by flow cytometry.

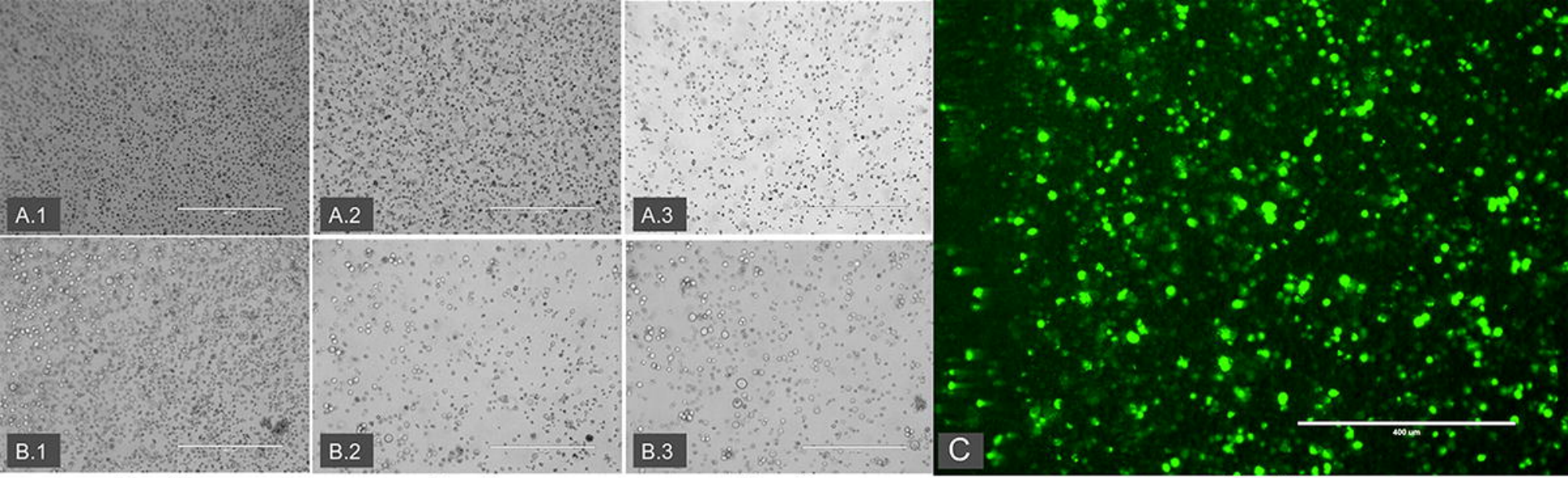
Passage No.	**Control mean MFI**	**K562-1634 mean MFI**	**Mean fold Change**
08	35524.43	8263.72	4.30
17	38175.67	9489.39	4.02
36	24464.225	13560.96	1.80

*K562-1634 denotes the K562 cells bearing the CRISPR plasmids. Numbers after '1634-' denote passage numbers.

Table II: Western blot analysis data for *pCRKL* and *pCRK*

Sample	***β-tubulin***	***pCRKL***	***pCRKL* ratio**	**Knockdown**	***pCRK***	***pCRK* ratio**	**Knockdown**
K562-1634-17	1.19	0.21	0.16	6.25x	0.46	0.28	4.10x
K562-1634-36	1.14	0.69	0.54	1.85x	0.89	0.58	2.00x
K562-1634-08	1.05	0.21	0.18	5.56x	0.40	0.28	4.12x
K562	1		0.97	Control		1.16	Control

*K562-1634 denotes the K562 cells bearing the CRISPR plasmids. Numbers after 'K562-1634-' denote passage numbers.


A.1
A.2
A.3
B.1
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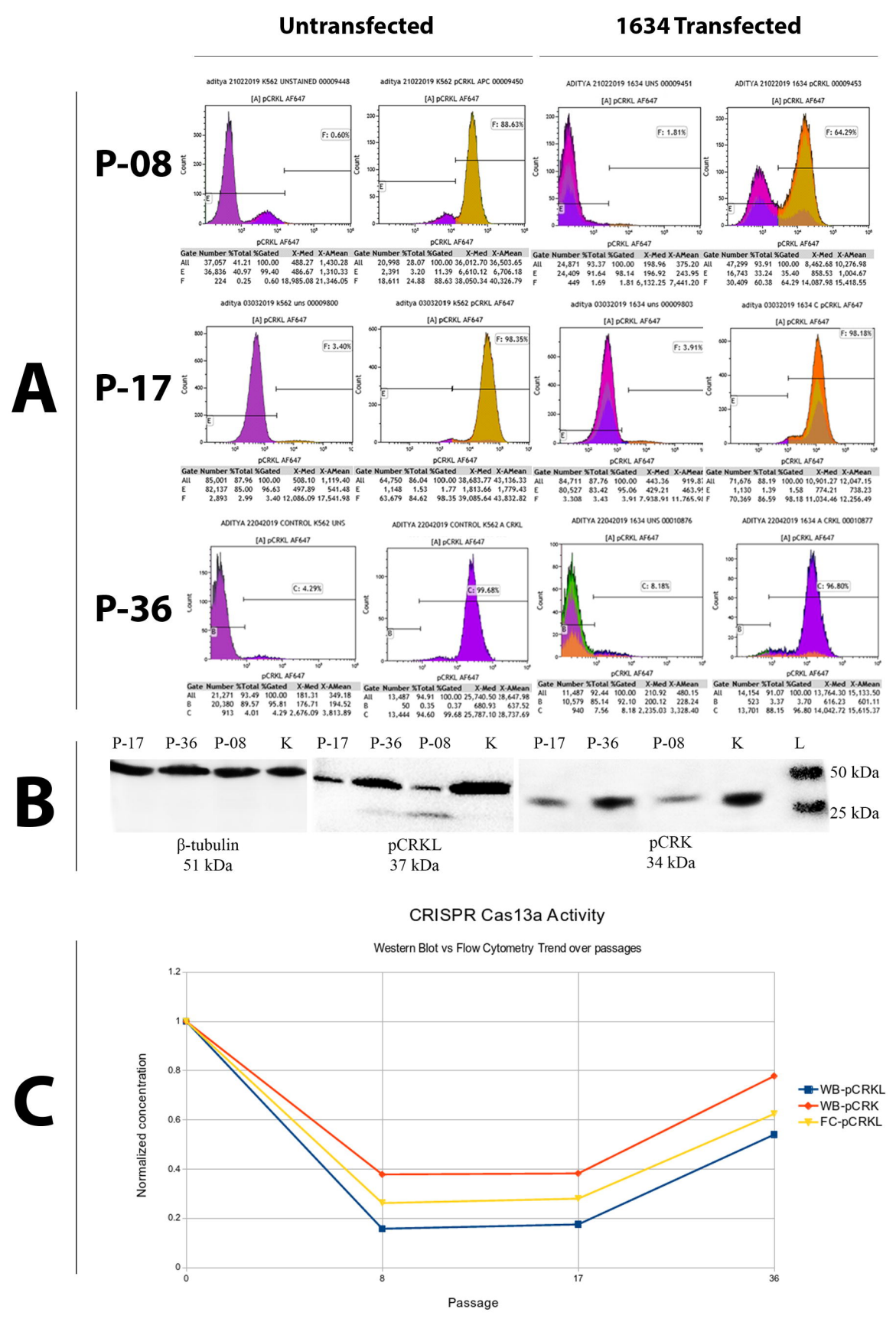
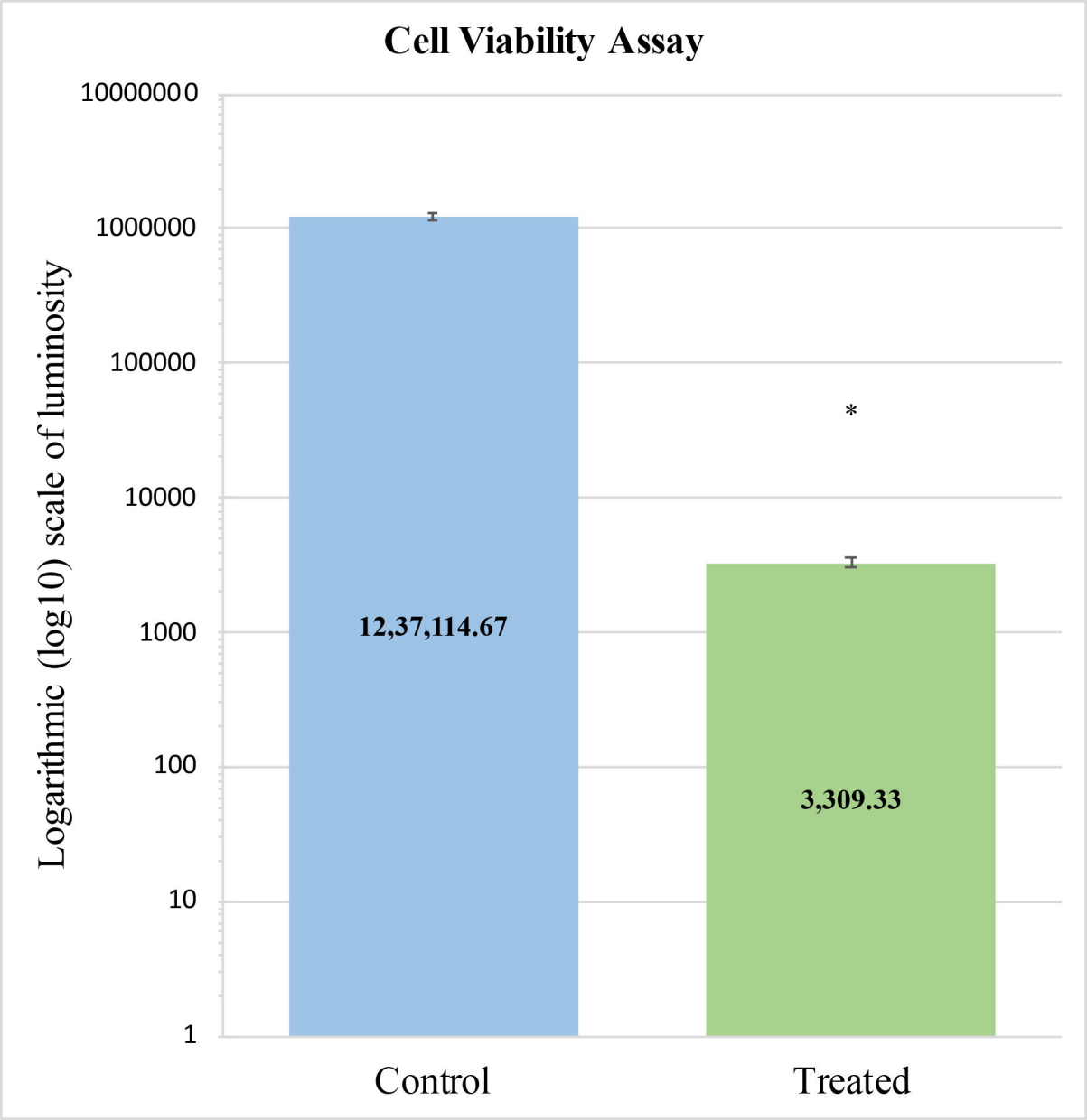
C
400 µm
Day 08
Day 10
Day 12
Day 16


Untransfected
1634 Transfected
A
P-08
P-17
P-36
B
P-17 P-36 P-08 K P-17 P-36 P-08 K P-17 P-36 P-08 K L
50 kDa
25 kDa
β-tubulin
51 kDa
pCRKL
37 kDa
pCRK
34 kDa
C
CRISPR Cas13a Activity
Western Blot vs Flow Cytometry Trend over passages
Normalized concentration
Passage
WB-pCRKL
WB-pCRK
FC-pCRKL


Cell Viability Assay
Logarithmic (log10) scale of luminosity
10000000
1000000
100000
10000
1000
100
10
1
*
12,37,114.67
3,309.33
Control
Treated