JGV Papers in Press. Published April 8, 2015 as doi:10.1099/vir.0.000139

Journal of General Virology

Inhibition of HIV-1 infection of primary CD4+ T cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9

--Manuscript Draft--

Manuscript Number:	VIR-D-15-00028R1
Full Title:	Inhibition of HIV-1 infection of primary CD4+ T cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9
Short Title:	CCR5 editing by adenovirus-delivered CRISPR/Cas9
Article Type:	Standard
Section/Category:	Animal - Retroviruses
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Abstract:	CCR5 serves as an essential coreceptor for HIV-1 entry and individuals with a CCR5Δ32 variant appears to be healthy, making CCR5 an attractive target for control of HIV-1 infection. The CRISPR/Cas9, that functions as a naturally-existed adaptive immune system in prokaryotes, has been recently harnessed as a novel nuclease system for genome editing in mammalian cells. Although CRISPR/Cas9 can be readily delivered into cell lines, due to the large size of Cas9 protein, efficient delivery of CCR5-targeting CRISPR/Cas9 components into primary cells, including CD4+ T cells, the primary target for HIV-1 infection in vivo, remains a challenge. In the current study, following design of a panel of top-ranked single-guided RNA (sgRNA) targeting the open reading frame of CCR5, we demonstrate that CRISPR/Cas9 can efficiently mediate the editing of CCR5 locus in cell lines, resulting in the knockout of CCR5 expression on cell surface. Next-generation sequencing revealed that various mutations were introduced around the predicted cleavage site of CCR5. For each of the three most effective sgRNAs that we analyzed, no significant off-target effects were detected at the 15 top-scored potential sites. More importantly, by constructing chimeric Ad5F35 adenoviruses carrying CRISPR/Cas9 components, we efficiently transduced primary CD4+ T lymphocytes and disrupted CCR5 expression, and the positively transduced cells were conferred with HIV-1 resistance. To our knowledge, this is the first study establishing HIV-1 resistance in primary CD4+ T cells utilizing adenovirus-delivered CRISPR/Cas9.

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Inhibition of HIV-1 infection of primary CD4$^+$ T cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9

Running title: CCR5 editing by adenovirus-delivered CRISPR/Cas9

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Key words: HIV-1; CCR5; adenovirus; CRISPR/Cas9; primary CD4$^+$ T cells

Word count: summary: 231; main text: 5497

Figures: 6; Supplementary files: 2

Summary

CCR5 serves as an essential coreceptor for HIV-1 entry and individuals with a $CCR5^{\Delta 32}$ variant appears to be healthy, making CCR5 an attractive target for control of HIV-1 infection. The CRISPR/Cas9, that functions as a naturally-existed adaptive immune system in prokaryotes, has been recently harnessed as a novel nuclease system for genome editing in mammalian cells. Although CRISPR/Cas9 can be readily delivered into cell lines, due to the large size of Cas9 protein, efficient delivery of CCR5-targeting CRISPR/Cas9 components into primary cells, including $CD4^+$ T cells, the primary target for HIV-1 infection *in vivo*, remains a challenge. In the current study, following design of a panel of top-ranked single-guided RNA (sgRNA) targeting the open reading frame of *CCR5*, we demonstrate that CRISPR/Cas9 can efficiently mediate the editing of *CCR5* locus in cell lines, resulting in the knockout of CCR5 expression on cell surface. Next-generation sequencing revealed that various mutations were introduced around the predicted cleavage site of *CCR5*. For each of the three most effective sgRNAs that we analyzed, no significant off-target effects were detected at the 15 top-scored potential sites. More importantly, by constructing chimeric Ad5F35 adenoviruses carrying CRISPR/Cas9 components, we efficiently transduced primary $CD4^+$ T lymphocytes and disrupted CCR5 expression, and the positively transduced cells were conferred with HIV-1 resistance. To our knowledge, this is the first study establishing HIV-1 resistance in primary $CD4^+$ T cells utilizing adenovirus-delivered CRISPR/Cas9.

Introduction

More than 33 years have elapsed (CDC-USA, 1981) since the first reported case of AIDS. Although the introduction of effective antiretroviral therapy (ART) has resulted in huge reductions in rates of HIV-1 illness, HIV-1 infection remains incurable. In addition, ART encounters a number of challenges, including a persistent latent viral reservoir, the requirement for lifelong adherence, and the potential development of drug resistance and toxicity (Chun *et al*., 2008; Corbeau & Reynes, 2011; Kent *et al*., 2013; Richman *et al*., 2009; Sendagire *et al*., 2009). Thus the development of novel therapeutic methods that may enhance current therapeutic options and even lead to curative approaches would significantly expand our portfolio of strategies to counteract HIV-1/AIDS.

Nuclease mediated-genome editing represents one promising strategy for HIV-1 therapy. The earlier developed nucleases including zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) recognize genome locus based on protein-DNA interactions (Mak *et al*., 2012; Pavletich & Pabo, 1991). By construction a tandem array of various artificially engineered modules, ZFN and TALEN can target virtually any DNA sequence (Boch *et al*., 2009; Urnov *et al*., 2005). However, these two technologies to some extent present limitations and remain not only technically complex but also laborious and time-consuming to manipulate. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein 9 (Cas9), derived from a type II CRISPR/Cas system naturally

existant in bacteria, has been harnessed as a novel nuclease tool to mediate genome editing in mammalian cells (Cong *et al*., 2013; Mali *et al*., 2013b). By simple delivery of two essential components, a human codon-optimized Cas9 protein and a single guided RNA (sgRNA), the formed CRISPR/Cas9 complex can be programmed to target any genomic locus followed by a 5'-protospacer adjacent motif (PAM) sequence of NGG, with the specificity determined by the sgRNA containing a 20-nt guide sequence complementary to the genome locus of interest (Cong *et al*., 2013; Mali *et al*., 2013b). Upon the guidance of sgRNA, Cas9 protein is programmed to cleave the targeted DNA at each strand, resulting in double-stranded breaks (DSBs) that trigger cellular repair mechanisms. In eukaryotes, the DSBs are more commonly repaired by the mechanism of error-prone non-homologous end joining (NHEJ), therefore generating sequence changes, for instances, insertions and deletions (indels) around the DSBs (Kim & Kim, 2014). Due to simplicity of manipulation and versatility, CRISPR/Cas9 system has been utilized as an attractive tool for various applications, such as genome-wide screening (Shalem *et al*., 2014; Zhou *et al*., 2014), gene repression and activation (Cheng *et al*., 2013; Doench *et al*., 2014; Gilbert *et al*., 2014), targeted fluorescence imaging (Tanenbaum *et al*., 2014) and novel approaches against pathogens including HBV (Lin *et al*., 2014a; Seeger & Sohn, 2014), HPV (Kennedy *et al*., 2014), EBV (Wang & Quake, 2014; Yuen *et al*., 2014), malaria (Wagner *et al*., 2014) and HIV-1 (Ebina *et al*., 2013; Hu *et al*., 2014; Ye *et al*., 2014).

Using a pair of sgRNAs targeting the long terminal repeat of HIV-1, it was shown that

HIV-1 provirus can be removed from the genome of infected cell lines (Ebina *et al*., 2013; Hu *et al*., 2014). By combining TALEN or CRISPR/Cas9 with PiggyBac technology, researches have generated induced-pluripotent stem cells (iPSC) homozygous for the naturally occurring $CCR5^{\Delta 32}$ variant resistant to HIV-1 infection (Ye *et al*., 2014). However, owing to the large size of Cas9 coding sequence with a total length more than 5 kb when combining with sgRNA, promoters and other essential elements for efficient expression, delivery of CRISPR/Cas9 components into primary cells, for instance, $CD4^+$ T lymphocytes, the primary target for HIV-1 infection *in vivo*, remains challenging. In the current study, after optimized design and screening of a panel of sgRNAs in cell lines, we obtained several sgRNA sequences with high potency to target CCR5. By utilizing a chimeric adenoviral vector, we demonstrated the efficacy of CCR5 editing and established HIV-1 resistance in primary $CD4^+$ T cells.

Results

Design of sgRNA guide sequences

The open reading frame (ORF) of HIV-1 coreceptor CCR5 is located on the fourth or last exon of *CCR5* gene, which maps to the short arm of chromosome 3 at position 21.31 (3p21.31), with base pair range from 46,370,854 to 46,376,206 of the forward strand based on GRCh38 coordinates (Fig. 1). The single guided RNA (sgRNA) was designed at the online platform http://crispr.mit.edu/ (Hsu *et al*., 2013). Based on the presence of protospacer adjacent motif (PAM) with a 5'-NGG sequence, a total of 124 possible sgRNAs can be designed to target the 1059 bp-long *CCR5* ORF or its complementary strand (Table S1). CRISPR/Cas9 was found to tolerate mismatches (MMs) between the sgRNA sequence and recognition site and the property of MMs, and in particular the number and location of MMs were shown to affect off-target effects (OTEs) (Hsu *et al*., 2013; Kuscu *et al*., 2014). Therefore, we further selected sgRNAs among the 20 top-ranked sgRNAs of the 124 candidates by applying the following rules: 1) MMs >= 2 bp; 2) for MMs = 2 bp, both locate in the PAM-proximal half; 3) for MMs = 3 bp, at least 1 bp locates in the PAM-proximal half; and 4) for overlapping sgRNAs with only 1 bp difference, choose the sgRNA with highest score. These rules were artificially set up mainly on the principle of increasing MMs, particularly MMs in the PAM-proximal half of the guide sequence, and diversity of the targeting region of our designed sgRNAs. As a result, 8 top-ranked sgRNAs, designated as sgR5-3 to 10, were generated (Fig. 1 and Table S2). 2 previously reported sgRNAs targeting CCR5 (sgR5-1 and 2) (Cradick *et al*., 2013)

and a non-targeting sgRNA (sgNeg) (Shalem *et al*., 2014) were employed as positive and negative controls, respectively.

Efficient editing of *CCR5* locus by the RNA-guided Cas9 nuclease

sgRNA-guided Cas9 cleaves target DNA at both strands and generates DSBs which commonly trigger the NHEJ and result in indels (Cong *et al*., 2013). We tested the on-target efficacy of the designed sgRNAs to guide editing of *CCR5* locus using T7 endonuclease I (T7EI) mutation detection assay. The HeLa-derived TZM-bl cells were infected with LentiCRISPR expressing Cas9 and sgRNA, followed by extraction of genomic DNA and amplification of a 1228 bp encompassing *CCR5* ORF (Table S3). T7EI analysis of the amplicons showed that all the 10 sgRNAs effectively guided CCR5 editing with various efficacies, while the scrambled sgRNA (sgNeg) guided no detectable editing. The top 2 effective sgRNAs, sgR5-5 and 8, resulted in 74.1% and 63.8% indels in *CCR5* allele, respectively. In contrast, the least effective sgR5-9 and 7 generated 27.1% and 30.8% indels, respectively (Fig. 2a). Of interest, the sgRNAs exhibiting higher editing efficacy mainly locate in the region from nt 254 to 315 of *CCR5* ORF. However, using chromatin immunoprecipitation assay, we did not find significant difference in terms of binding affinity between sgR5-5 and sgR5-7, of which one targets the region between nt 254 and 315 of *CCR5* ORF and the other outside this region (Fig. S1).

To determine the detailed sequence changes resulted from CRISPR/Cas9-mediated

gene editing, we amplified the genomic segment encompassing the predicted cleavage site and sequenced the amplicons by deep sequencing. Results showed that diverse changes were generated (Fig. 2b and Fig. S2), consistent with previous findings (Cong *et al*., 2013). For sgR5-5, various mutations from 1-bp insertion to 51-bp deletion were frequently read out. The mutation with the most reads number was a 16-bp deletion at the predicted cleavage site (Fig. 2b). Similar results were observed for sgR5-8, while the mutation with the most reads number was an 11-bp deletion at the predicted cleavage site (Fig. S2). The above results collectively demonstrated that CRISPR/Cas9 is an efficient tool to edit *CCR5* locus.

To determine the specificity of our designed sgRNAs, we measured OTEs of sgR5-5, 6 and 8, which exhibited highest efficacy of on-target editing. For each of the three sgRNAs, the 15 top-ranked off-target sites in whole human genome were analyzed (Table S3 and S4). Approximate 500-bp segments spanning the potential off-target sites were amplified from genomic DNA of TZM-bl cells and subjected to T7EI analysis. No significant cleavage was detected for all of these potential off-target sites (Fig. S3). Furthermore, because *CCR2* shares high extent of sequence homology with *CCR5*, we tested OTEs of the three sgRNAs in *CCR2*. For sgR5-6, the off-target sites in *CCR2* are the same as OTE-56-15 (Fig. S2, Table S3, S4 and S5). For sgR5-5 and 8, we observed no significant cleavage at their homologous regions in *CCR2* (Fig. S4 and Table S5), demonstrating high specificity of Cas9 nuclease directed by the three sgRNAs.

HIV-1 resistance in susceptible cell lines conferred by disruption of CCR5 expression

Next, we measured CCR5 protein expression on cell surface following CRISPR/Cas9-mediated gene editing. Consistent with T7EI cleavage assay, CCR5 expression on TZM-bl cells assayed by flow cytometry (FCM) was found to be correspondingly disrupted by the panel of sgRNAs (Fig. 3a). Maximum disruption was observed on cells edited by sgR5-5 and 8 which exhibited most potent capability in mediating *CCR5* cleavage. To determine the cell type dependence of these results, we further measured CCR5 disruption on CHO-CCR5, a CHO-K1 derivative with stable CCR5 expression (Hu *et al*., 2005), and C8166-CCR5 cells, a human T lymphocyte cell line supporting HIV-1 growth (Soda *et al*., 1999). Similarly, CCR5 expression on CHO-CCR5 and C8166-CCR5 cells were abrogated by sgR5-5 and 8 to the similar extent as that observed on TZM-bl cells (Fig. 3a).

We then confirmed if disruption of coreceptor CCR5 expression by CRISPR/Cas9 conferred HIV-1 resistance. TZM-bl cells edited by sgR5-5 and 8 were subjected to HIV-1 BaL (R5-tropic) infection, followed by luciferase reporter assay to quantify the infection. Results revealed that BaL infection in these cells were largely decreased compared to that in cells edited by sgNeg or without editing (Fig. 3b), consistent with the indispensable role of CCR5 in R-tropic HIV-1 infection (Alkhatib *et al*., 1996). As expected, infection with NL4-3, an X4-tropic HIV-1, was not affected whether the

cells were CCR5 edited or not (Fig. 3b).

Delivery of CRISPR/Cas9 system by a chimeric Ad5F35 adenoviral vector

HIV-1 mainly targets $CD4^+$ T cells *in vivo*. Although lentiviral vector transduces T cells with high efficacy, the capacity of lentivirus is relatively small, resulting in low titer of lentivirus carrying the large CRISPR/Cas9 insert. In addition, the integrative property of lentiviral vector is of critical concern in therapeutic applications. We thus sought to deliver the CRISPR/Cas9 components into primary $CD4^+$ T cells using a chimeric Ad5/F35 adenoviral vector, which has been shown to transduce $CD34^+$ hematopoietic cells with high efficacy (Gaggar *et al*., 2003; Shayakhmetov *et al*., 2000). We constructed the recombinant adenoviral vectors pAd5F35.Cas9.sgR5-5/8/sgNeg.EGFP (designated as pAd5F35.$g^{5/8/N}$Cas9) carrying the 5.9-kb CRISPR/Cas9 and an EGFP reporter (Fig. 4a and Table S6). The 35.2-kb genome size of pAd5F35.gCas9 was smaller than the theoretical package capacity of E1E3-deleted adenoviral vectors (Bett *et al*., 1994). Recombinant Ad5F35.gCas9 virus was rescued by transient transfection of HEK293 cells. Apparent cytopathic effects were observed at 10-16 days post-transfection (Fig. 4b). Purified viral stocks with high titer (up to 10^{10} infectious viral particles ml^-) were obtained by CsCl banding following several rounds of amplification in HEK293 cells. Following infection of HEK293 cells with the purified Ad5F35.gCas9 viral stocks, Cas9 protein expression was detected (Fig. 4c), confirming that the large CRISPR/Cas9 components can be successfully packaged by Ad5F35 vector. TZM-bl cells

transduced with Ad5F35.g$^{5/8}$Cas9 exhibited significant CCR5 editing, while cells transduced with Ad5F35.EGFP (Ad5F35 carrying EGFP alone) or Ad5F35.g^{N}Cas9 showed no editing (Fig. 4d), validating the functionality of the recombinant viral stocks. Notably, our results showed that the efficacy of editing varied between adenovirus- and lentivirus-delivered CRISPR/Cas9 (Fig. 2a and 4d). This was likely resulted from the differences in transduction multiplicity of infection (MOI), integration property and antibiotic selection of these two approaches.

Gene editing of CCR5 in primary CD4$^+$ T cells by Ad5F35-delivered CRISPR/Cas9

We subsequently investigated the editing efficacy of the produced Ad5F35 viruses on CD4$^+$ T cells. Primary CD4$^+$ T cells were activated with PHA for 24 hours and then exposed to Ad5F35 viruses. The MOI was set as 30 and 100 according to previous reports (Perez *et al*., 2008; Shayakhmetov *et al*., 2000) and under these conditions no significant cytotoxic effect was observed by trypan blue staining. Transduction efficiency was monitored by GFP expression, which appeared as early as 24 hours post-transduction (p.t.), peaked at 4 days p.t. on average and remained nearly constant at least 10 days p.t. At peaked level, CD4$^+$ T cells transduced with Ad5F35.gCas9 at a MOI of 30 or 100 exhibited 24.6% (n = 9) or 30.3% (n = 3) of GFP expression correspondingly, while 45.3% (n = 3) of cells exposed to 30 MOI of Ad5F35.EGFP were GFP positive (Fig. 5a), comparable with previous reports (Shayakhmetov *et al*., 2000). Gene editing of CCR5 in the total cells exposed to Ad5F35.g^{5}Cas9 was

determined at various time points p.t. and maximum level of editing was achieved at approximate 8 days p.t (Fig. S5). Therefore, we further purified the positively transduced (GFP^+) cells at least 8 days p.t. by FCM sorting and analyzed CCR5 editing. Results showed that for either sgR5-5 or 8, more that 30% indels were generated in the *CCR5* allele derived from the GFP^+ cells, while no obvious indels were generated by the Ad5F35.g^NCas9 (Fig. 5b). Correspondingly, cell surface CCR5 expression was also significantly disrupted by Ad5F35.$g^{5/8}$Cas9 (Fig. 5c), but not Ad5F35.g^NCas9 or Ad5F35.EGFP. We further assayed potential OTEs in $CD4^+$ T cells. Utilizing the same approach as in TZM-bl cells, we tested the 15 top-ranked potential off-target sites for both Ad5F35.g^5Cas9 and Ad5F35.g^8Cas9 (MOI=30) and observed no significant OTEs.

Inhibition of HIV-1 infection of primary $CD4^+$ T cells by Ad5F35-delivered CRISPR/Cas9

To test if $CD4^+$ T cells with CCR5 edited by CRISPR/Cas9 gained HIV-1 resistance, the positively transduced $CD4^+$ T cells at least 8 days p.t. were sorted by FCM as described above and exposed to HIV-1 infection. Virus infection was determined by measurement of p24 in the culture supernatants. Results showed that at 2 ng p24 inoculum of HIV-1 BaL but not X4-tropic NL4-3, infection of $CD4^+$ T cells transduced with Ad5F35.$g^{5/8}$Cas9 was markedly decreased compared to that of cells transduced with Ad5F35.g^NCas9 or Ad5F35.EGFP (Fig. 6a and S6). When virus inoculum was increased to 10 ng of p24, comparable extent of inhibition was detected

at 7 days post-infection (Fig. 6b). Transmitted/founder (T/F) HIV-1 strains isolated very early after transmission are thought to be more physiologically relevant than laboratory-adapted strains (Keele *et al*., 2008). We examined if CRISPR/Cas9-mediated disruption of CCR5 also affected CH042, a T/F strain infection. Similar results were obtained (Fig. 6c), confirming the indispensable role of CCR5 for R5 HIV-1 infection.

Discussion

Although antiretroviral therapy is one of the most prominent achievements in HIV-1/AIDS research, AIDS remains an incurable disease (Lewin, 2013; Richman *et al*., 2009). Gene therapy represents one alternative approach for treatment of HIV-1 infection (DiGiusto *et al*., 2013; Herrera-Carrillo & Berkhout, 2014; Tebas *et al*., 2014). Here, we demonstrated that CRISPR/Cas9 can efficiently cut the *CCR5* locus, resulting in various sequence changes, and disrupted CCR5 expression. At the same time, the high on-target efficacy was not observed to associate with obvious off-target effects (OTEs). Importantly, we efficiently delivered the CRISPR/Cas9 components into primary $CD4^+$ T cells using recombinant adenoviral vectors, which inhibited HIV-1 infection in the positively transduced cells following CCR5 disruption.

Owing to the indispensable role of CCR5 for HIV-1 entry and the naturally presence of $CCR5^{\Delta 32}$ variant, a number of gene editing studies have targeted CCR5 by various approaches (Badia *et al*., 2014; Holt *et al*., 2010; Huang *et al*., 1996; Mussolino *et al*., 2014; Perez *et al*., 2008; Saito *et al*., 2014; Ye *et al*., 2014). In a recently finished Phase I clinical trial, autologous $CD4^+$ T cells with CCR5 modified by ZFN showed promise in alleviating the disease condition of some HIV-1 patients (Tebas *et al*., 2014). However, genome editing utilizing early-developed nucleases, including ZFN and TALEN, remains technically challenging, and the broad application of these technologies is consequently somewhat limited. The prokaryote-derived CRISPR/Cas9 system is a robust genome editing tool with versatile applications (Mali

et al., 2013a). In our study, among the panel of designed sgRNAs, we observed generally high levels of on-target cleavage of CCR5. However, the efficacy varied markedly for various sgRNAs, consistent with previous findings that the efficacy of editing is mainly determined by the guide sequence (Cong *et al*., 2013). One interesting finding is that, compared to other regions of *CCR5* ORF, there was a trend for sgRNAs targeting the region from nt 254 to 315 to exhibit higher efficacies of gene editing. Of interest, our ChIP results suggest that, at least for the sgRNAs tested, such higher efficacies are unlikely due to the difference of binding affinity between sgRNAs targeting this region and those targeting other regions. The underlying mechanisms and whether other genes also possess such hotspots for CRISPR/Cas9 targeting remains to be explored in future studies.

The sgRNA/Cas9 complex recognizes target sites based on the rule of Waston-Crick base pairing, conferring overall high specificity of the CRISPR/Cas9 system (Hsu *et al*., 2013; Smith *et al*., 2014; Veres *et al*., 2014). However, CRISPR/Cas9 was found to tolerate mismatches (MMs) between its sgRNA and target sites (Duan *et al*., 2014; Kuscu *et al*., 2014; Lin *et al*., 2014b). We selected the sgRNAs according to the guidelines of increasing the number and position of MMs, particularly MMs locating at the PAM-proximal half of the sgRNA which were shown to be critical for determining OTEs (Hsu *et al*., 2013). Among the total 45 top-ranked off-target sites (15 for each of the three most potent sgRNAs) tested, we observed no significant OTEs, confirming the specificity of CRISPR/Cas9 system. However, due to the

limited sites we analyzed and the restricted sensitivity of T7EI cleavage assay, lower incidents of OTEs for these sgRNAs at a genome-wide scale remain to be determined by more powerful approaches, such as ultra-deep next-generation sequencing of the whole genome (Hu *et al*., 2014; Smith *et al*., 2014; Veres *et al*., 2014). Studies aimed to develop modified CRISPR/Cas9 systems are also promising to further improve the on-target fidelity of CRISPR/Cas9 (Guilinger *et al*., 2014; Qi *et al*., 2013; Ran *et al*., 2013).

Generally, delivery of CRISPR/Cas9 components into cell lines can be readily achieved. In the process of this manuscript submission, by delivery of CRISPR/Cas9 utilizing an integrative lentiviral vector Wang et al. reported that targeting of CCR5 rendered CEMss-CCR5, a $CD4^+$ T cell line, resistant to HIV-1 infection (Wang *et al*., 2014). However, efficient delivery of the large CRISPR/Cas9 components into primary cells remains challenging. Another recent report utilized the approach of nucleofection to deliver sgRNA and Cas9 into hematopoietic stem cell and primary $CD4^+$ T cells (Mandal *et al*., 2014). Although nucleofection is a direct delivery approach, its efficacy and toxicity effects on primary cells restrict its application. We therefore sought to utilize relatively safe viral vectors which are widely adopted by gene therapy studies (Kootstra & Verma, 2003; Tebas *et al*., 2014). Considering that 1) only short-term expression is desired for nuclease to play its role (Cong *et al*., 2013; Mali *et al*., 2013b); 2) high capacity is required to carry the large CRISPR/Cas9 components; 3) side effects such as oncogenic potential should be avoided; and 4)

tropism for hematopoietic cell lineage is needed to efficiently transduce HIV-1 target cells, we utilized the chimeric adenoviral vector Ad5F35 as the vehicle to deliver the CRISPR/Cas9 components. Our results demonstrated that CRISPR/Cas9 components reaching a length of 5.9 kb when combining with a GFP reporter gene can be successfully packaged into the recombinant Ad5F35 adenovirus, confirming the large capacity of adenoviral vectors (Bett *et al*., 1994; Cheng *et al*., 2014; Maggio *et al*., 2014). Ad5F35 carrying CRISPR/Cas9 efficiently transduced primary $CD4^+$ T cells and disrupted CCR5 expression. However, for the positively transduced GFP^+ cells, we observed that the level of CCR5 editing appeared to be relatively lower in $CD4^+$ T cells compared to that in TZM-bl cells. This is probably resulted from various efficacies of expression and activity of the CRISPR/Cas9 components between primary cells and cell lines, or other yet-to-be-defined mechanisms. Nevertheless, CRISPR/Cas9-mediated disruption of CCR5 expression markedly reduced susceptibility of $CD4^+$ T cells to HIV-1 infection, including BaL and a transmitted/founder strain. Whether engraftment of humanized mouse or/and primate animals with the modified $CD4^+$ T cells could be conferred with HIV-1 resistance warrants further investigations.

Here, we targeted CCR5 to confer R5-tropic HIV-1 resistance in primary lymphocytes. Although CCR5 is essential for the entry of R5-tropic viruses and partial ablation of CCR5 may provide clinical benefit for HIV-1 patients, it is likely that targeting CCR5 may drive HIV-1 coreceptor switch (Poveda, 2015). While the driving force and

molecular mechanism of coreceptor switch remain elusive, antiretroviral treatments with CCR5 antagonists tend to select for R5X4 or X4-tropic variants (Fatkenheuer *et al*., 2008; Moncunill *et al*., 2008; Mosier, 2009). A recent study has reported that a shift from R5 tropic to X4 tropic HIV-1 variants occurred in a patient following stem-cell transplantation with $CCR5^{\Delta 32}$ mutation (Kordelas *et al*., 2014), further highlighting the importance of coreceptor switch or adaptation in gene therapy targeting CCR5. Nevertheless, by delivery of CRISPR/Cas9 with two sgRNAs simultaneously targeting CCR5 and CXCR4, or CCR5 and viral genes essential for HIV-1 infection, it is theoretically applicable in preventing coreceptor adaptation and virus infection more effectively.

$CD4^+$ T cells or hematopoietic stem cells (HSC) are two branches for delivery of HIV-1 gene therapy (Savkovic *et al*., 2014). The current study established HIV-1 resistance in primary $CD4^+$ T cells mainly because its availability in terms of collection and expansion and safety when testing in clinical trials (Stan & Zaia, 2014). In contrast, gene therapy utilizing Ad5F35 transduced HSC is more challenging. Besides the relative complexity for handling HSC, one of the most critical challenges in clinical application is that allogeneic HSC transplantation usually accompanies with severe immune rejection which therefore requires prolonged immune-suppressive treatments (Stan & Zaia, 2014). However, Ad5F35 modified HSC have advantage over $CD4^+$ T cells in some aspects. Like the Berlin patient, if the HSC transplantation is conducted successfully, one time of therapy may enable

long-term control and even cure of HIV-1 infection (DiGiusto *et al*., 2013; Holt *et al*., 2010; Hutter *et al*., 2009).

In summary, here we conducted CCR5 gene editing studies to inhibit HIV-1 infection by utilizing the recently harnessed CRISPR/Cas9 system. Following optimized sgRNA design and selection, Cas9 nuclease can cleave *CCR5* genomic locus around the predicted cleavage site with high efficacy and specificity. Importantly, by utilizing a chimeric Ad5F35 adenoviral vector, we successfully packaged recombinant Ad5F35 adenovirus carrying CRISPR/Cas9 components and transduced primary $CD4^+$ T cells. The positively transduced cells with disrupted CCR5 expression were resultantly conferred with HIV-1 resistance. Considering the properties of versatility and being easy to manipulation, our results highlight the potential of utilizing CRISPR/Cas9 in HIV-1 gene therapy.

Methods

Plasmids, antibodies and cell lines

pLentiCRISPR.v2, pAdeasy-1, pShuttle-CMV and pLentiLox3.7 (pLL3.7) were from Addgene. pNL4-3 BaL^+ (R5-tropic, containing the backbone of NL4-3 and the Env of BaL, referred to as BaL hereafter) and pNL4-3 (X4-tropic), as well as TZM-bl and C8166-CCR5 cell lines were from NIH AIDS Research & Reference Reagent Program, Division of AIDS, NIH. pCH042, a transmitted/founder HIV-1 molecular clone were kindly provided by Professor Beatrice H. Hahn (Parrish *et al*., 2013). Anti-CCR5-PE (clone 2D7) and matched isotype control antibodies were from BD Pharmingen. Rabbit anti-Cas9 polyclonal antibody was purchased from Hangzhou HuaAn Biotechnology Co. Ltd, and the internal control antibody (anti-β-actin) was purchased from Santa Cruz. CHO-CCR5, a CHO-K1 cell line with stable CCR5 expression was described previously (Hu *et al*., 2005).

Lentivirus preparation

The designed sgRNAs (Table S2) were constructed into pLentiCRISPR.v2 as described (Shalem *et al*., 2014). Lentiviruses were produced by co-transfection of HEK293T cells with sgRNA-inserted pLentiCRISPR.v2, psPAX2 and pMG2.G, followed by concentration of the virus stocks by ultracentrifugation. Virus stocks were titrated by determining the cell number resistant to puromycin selection following infection of HEK293T cells. (Shalem *et al*., 2014; Tiscornia *et al*., 2006).

Cells were transduced with lentiviruses (MOI = 1) in the presence of polybrene for 24 hours. 48 hours post-transduction (p.t.), puromycin was presented at a concentration to kill untransduced cells within 2-3 days. After removal of puromycin, tranduced cells were further cultured until downstream analysis.

T7EI cleavage assay

Fragments of interest were amplified from genomic DNA by flanking primers (Table S3, S4 and S5). The amplicons were purified, annealed and subjected to T7 endonuclease I digestion to determine the levels of gene editing as described previously (Ramakrishna *et al*., 2014). Indels% (= 100% × (1-$\sqrt[2]{1-(\text{amount of cut DNA}/\text{total DNA})}$)) (Cong *et al*., 2013).

Production of recombinant adenoviruses

The shuttle plasmid pShuttle.CRISPR.EGFP was constructed by inserting CRISPR/Cas9 components with an EGFP reporter into the CMV-removed pShuttle-CMV. Ad5F35 adenoviral backbone was constructed by exchange the fiber shaft and knob region of pAdeasy-1 with that from Ad35 as previously reported (Shayakhmetov *et al*., 2000). Recombinant Ad5F35 adenoviral vectors carrying CRISPR/Cas9 (i.e., pAd5F35.Cas9.sgR5-5/8/sgNeg.EGFP, designated as pAd5F35.g$^{5/8/N}$Cas9 or pAd5F35.gCas9) were generated in BJ5183 cells via homologous recombination (Luo *et al*., 2007). Recombinant adenoviral viruses were produced by transfection of HEK293 cells, followed by scale-up propagation and

purification via CsCl banding (Luo *et al*., 2007). Detailed cloning strategies, used primers and virus production methods are available in the supplementary materials.

Adenovirus transduction

Adenoviral stocks were thawed in a 37°C water bath and taken out immediately once the ice was dissolved. For cell lines, the cells were seeded in 6-well plates overnight to be 30-50% confluent at the time of virus inoculation. 30 MOI of Ad5F35 viruses were added to each well and incubated for 2-3 hours followed by exchange with fresh medium. For primary $CD4^+$ T cells, indicated amount of viruses (30 or 100 MOI) were used to transduce 5×10^5 of cells which were plated in 24-well plates at the time of transduction in a total volume of 200 μL medium per well. 2 hours p.t., additional 300 μL medium was added and the cells were incubated for another 4 hours. Thereafter, the culture medium was removed and the cells were washed once with FBS-free medium to further remove the unbound viruses. The transduced cells were cultured at 37°C to allow transgene expression and/or gene editing until downstream analysis.

HIV-1 production and infection

HIV-1 was produced by transfecting plasmid of HIV-1 infectious clone into 293T cells using Lipofectamine 2000 according to the manufacturer's instructions. All viral stocks were titrated by p24 ELISA (Beckman Coulter). Virus infection of $CD4^+$ T cells was conducted in 96-well plates. In each well, 1×10^5 cells were infected with

indicated amounts of viruses for 3 hours at 37°C, followed by extensive washes to remove unbound viruses. Cells were resuspended in 200 μL of medium and cultured at 37°C. The cultured supernatants were collected at indicated days post-infection, lysed with 1% (v/v) Triton X-100 and subjected to p24 quantification.

For virus infection of TZM-bl cells, the cells, pre-seeded into 96-well plate, were infected with HIV-1 for 48 hours, followed by determination of luciferase activity in the cell lysates using the Modulus II Microplate Multimode Reader (Promega) (Jin *et al*., 2014).

See supplementary materials for more experimental procedures.

Acknowledgements

This work was supported by Ministry of Science and Technology of China Grants 2010CB530100, 2013ZX10001005-003-002 and 2012ZX10001006-002, National Natural Science Foundation of China Grant 81273250, and the Hotung Trust.

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Fig. 1. Diagram of the composition and genomic location of *CCR5* and the designed sgRNA sequences. The genomic location of *CCR5* gene maps to the short arm of chromosome 3, with a base pair range from 46,370,854 to 46,376,206. The four exons (magenta boxes) and two introns (magenta lines) of *CCR5* gene are shown. The open reading frame (ORF) of *CCR5* locates in the fourth exon. The blue bars represent designed sgRNA sequences targeting specific sites of *CCR5* ORF, as shown by nucleotide sequences. The orange bars, corresponding to the red NGG sequences in *CCR5* ORF, denote the protospacer adjacent motif (PAM) region. The ORF is numbered starting from the start condon.

Fig. 2. Efficacy of on-target editing of *CCR5* locus by sgRNA-guided Cas9 nuclease. (a) Analysis of gene editing efficacy in TZM-bl cells by T7EI cleavage assay. Following transduction of TZM-bl cells with LentiCRISPR expressing Cas9 and various sgRNAs targeting *CCR5* (sgR5-1 to -10) or non-targeting sgRNA (sgNeg) (MOI = 1), the genomic DNA of the positively transduced cells were extracted and used as template to amplify a 1228-bp fragment encompassing *CCR5* ORF. The amplicons were purified, denatured and self-annealed, followed by T7EI digestion and agarose gel analysis. Mock, the sample from untreated cells. One representative out of three independent experiments is shown. (b) Alignment of the sgR5-5-edited sequences from deep sequencing. The amplicons derived from the genomic DNA of sgR5-5-edited cells were appended with barcode and subjected to deep sequencing by Illumina MiSeq system. Acquired sequences with high reads number (top 32) were

aligned and shown. The reads numbers of different sequences were ranked and indicated by the background color scale, with red and light pink represent the highest and the lowest reads number, respectively.

Fig. 3. Conferred HIV-1 resistance by disruption of CCR5 expression in susceptible cell lines. (a) Followed by transduction with lentivirus expressing Cas9 and various sgRNAs targeting *CCR5* or sgNeg, TZM-bl, CHO-CCR5 and C8166-CCR5 cells were stained with anti-CCR5-PE (clone 2D7) and assayed for CCR5 expression by FCM. (b) HIV-1 infection of TZM-bl cells following disruption of CCR5 expression. TZM-bl cells edited by sgR5-5, sgR5-8 or sgNeg-guided Cas9, or without editing were infected with 2 ng or 10 ng p24 of HIV-1 BaL (R5-tropic, left) or NL4-3 (X4-tropic, right). Virus infection was determined by luciferase activity assay and presented as percentage of infection. HIV-1 infection of TZM-bl cells without CCR5 editing was set as 100%. One representative out of three independent experiments is shown.

Fig. 4. Construction and validation of Ad5F35 adenovirus expressing CRISPR/Cas9. (a) Diagram of the recombinant adenoviral vector carrying CRISPR/Cas9 components. The circular map was created by Vector NTI Advance 11 (Invitrogen). Regions of CRISPR/Cas9 (referred to as gCas9) and the chimeric Ad5F35 fiber are highlighted. The arrangement of gCas9 components is shown on the top of the map. An EGFP was bicistronically expressed with Cas9 to track the

presence of the virus. The genome size of the recombinant vector (*Pac* I-linearized) is shown below the map. ψ, package signal. L/R-ITR, left/right-inverted terminal repeat. (b) A typical late cytopathic effect (CPE) of the recombinant virus Ad5F35.gCas9. Photos were taken at 15 days post-transfection of HEK293 cells with linearized pAd5F35.gCas9. Scale bars = 50 μM. (c) Cas9 expression in HEK293 cells after adenovirus infection. HEK293 cells were infected with Ad5F35.gCas9 for 3 days, followed by Western Blotting to detect Cas9 expression using a rabbit polyclonal antibody against Cas9. HEK293 cells infected with LentiCRISPR or Ad5F35.EGFP (Ad5F35 expressing EGFP alone), or without virus infection was served as positive, negative and mock controls, respectively. (d) T7EI cleavage assay of *CCR5* locus in TZM-bl cells following adenovirus infection. TZM-bl cells were mock infected or infected with various Ad5F35 viruses for at least 7 days, followed by T7EI assay as described in the legend of Fig. 2(a). Ad5F35.g$^{N/5/8}$Cas9 represents recombinant adenovirus expressing sgNeg, sgR5-5 or sgR5-8-guided Cas9. For (b)-(d), one representative out of three independent experiments is shown.

Fig. 5. Transduction and gene editing efficacy of Ad5F35 adenoviruses in primary CD4$^+$ T cells. (a) Transduction efficacy of Ad5F35 in primary CD4$^+$ T cells. CD4$^+$ T cells were stimulated with PHA and mock transduced or transduced with Ad5F35.EGFP (MOI = 30) or Ad5F35.gCas9 (MOI = 30 or 100). The dot plots shown are EGFP expression assayed by FCM at 8 days p.t. (b) Gene editing efficacy of *CCR5* locus in the positively transduced (GFP$^+$) CD4$^+$ T cells. The GFP$^+$ cells,

transduced with various Ad5F35 (MOI = 30), were obtained by FCM sorting, followed by T7EI cleavage assay as described in the legend of Fig. 2(a). (c) CCR5 expression on GFP^+ $CD4^+$ T cells. At 8 days p.t., $CD4^+$ T cells transduced with various Ad5F35 (MOI = 30) were stained with anti-CCR5-PE and subjected to FCM assay. Data shown are representative out of three independent experiments using $CD4^+$ T cells derived from different donors.

Fig. 6. HIV-1 infection of primary $CD4^+$ T cells following CRISPR/Cas9-mediated disruption of CCR5. (a) HIV-1 BaL infection of Ad5F35-positively transduced $CD4^+$ T cells. PHA-stimulated $CD4^+$ T cells were mock transduced or transduced with various Ad5F35 adenovirus (MOI = 30). The positively transduced GFP^+ cells were separated by FCM sorting and infected with HIV-1 BaL (2 ng p24 of virus). Half of the culture medium was collected at indicated days post-infection (p.i.), followed by measurement of p24 content. (b, c) HIV-1 BaL (b) and CH042 (c) infection of Ad5F35-positively transduced $CD4^+$ T cells. The experiment was carried out as in (a), except that two inoculum of 2 ng and 10 ng p24 of HIV-1 were conducted and p24 concentration at 7 days p.i. was shown. Each treatment was conducted in sextuplicate. Data shown (mean ± SD) are representative of three independent experiments using $CD4^+$ T cells derived from different donors.

Figure 1
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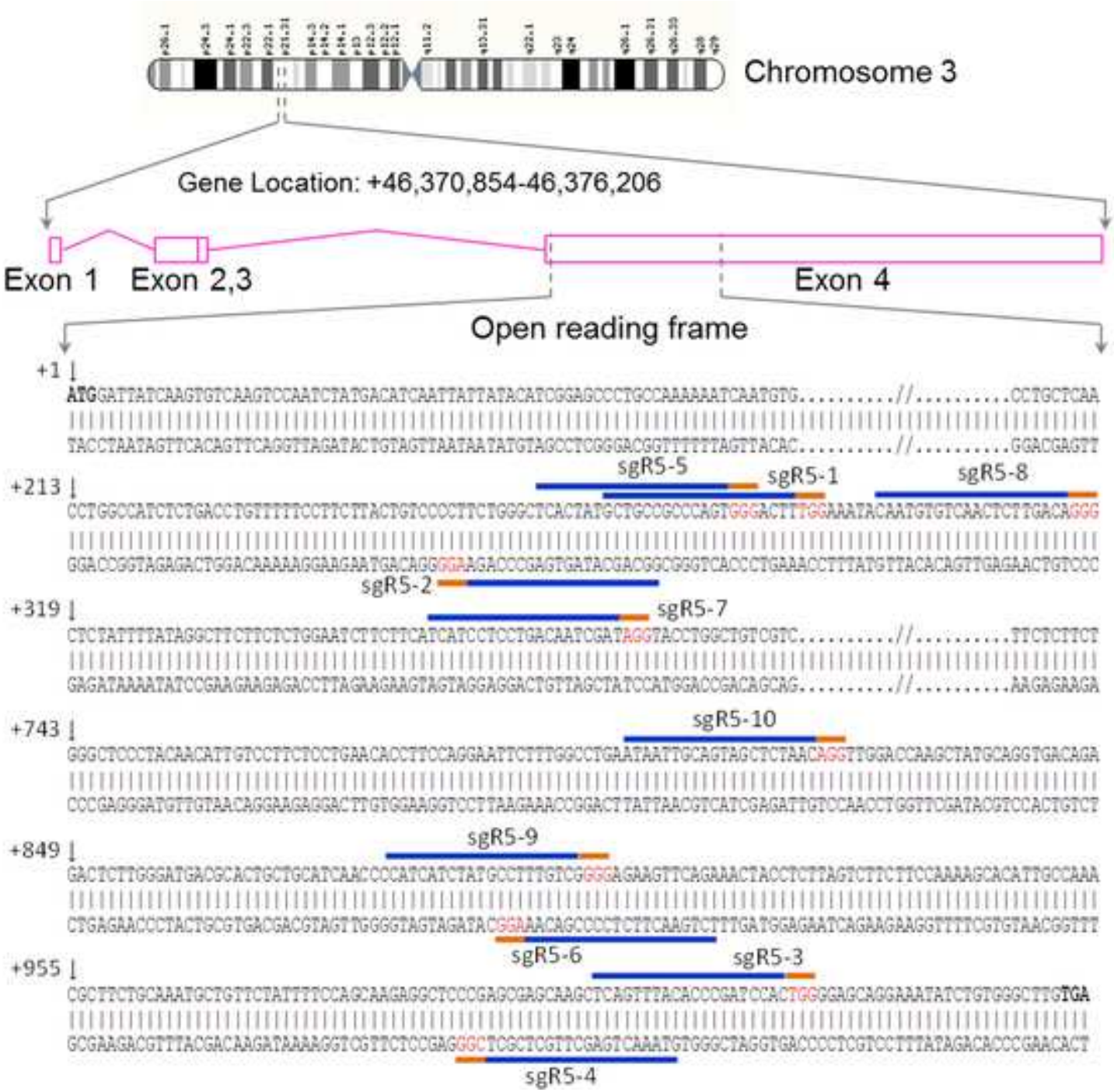


Figure 2

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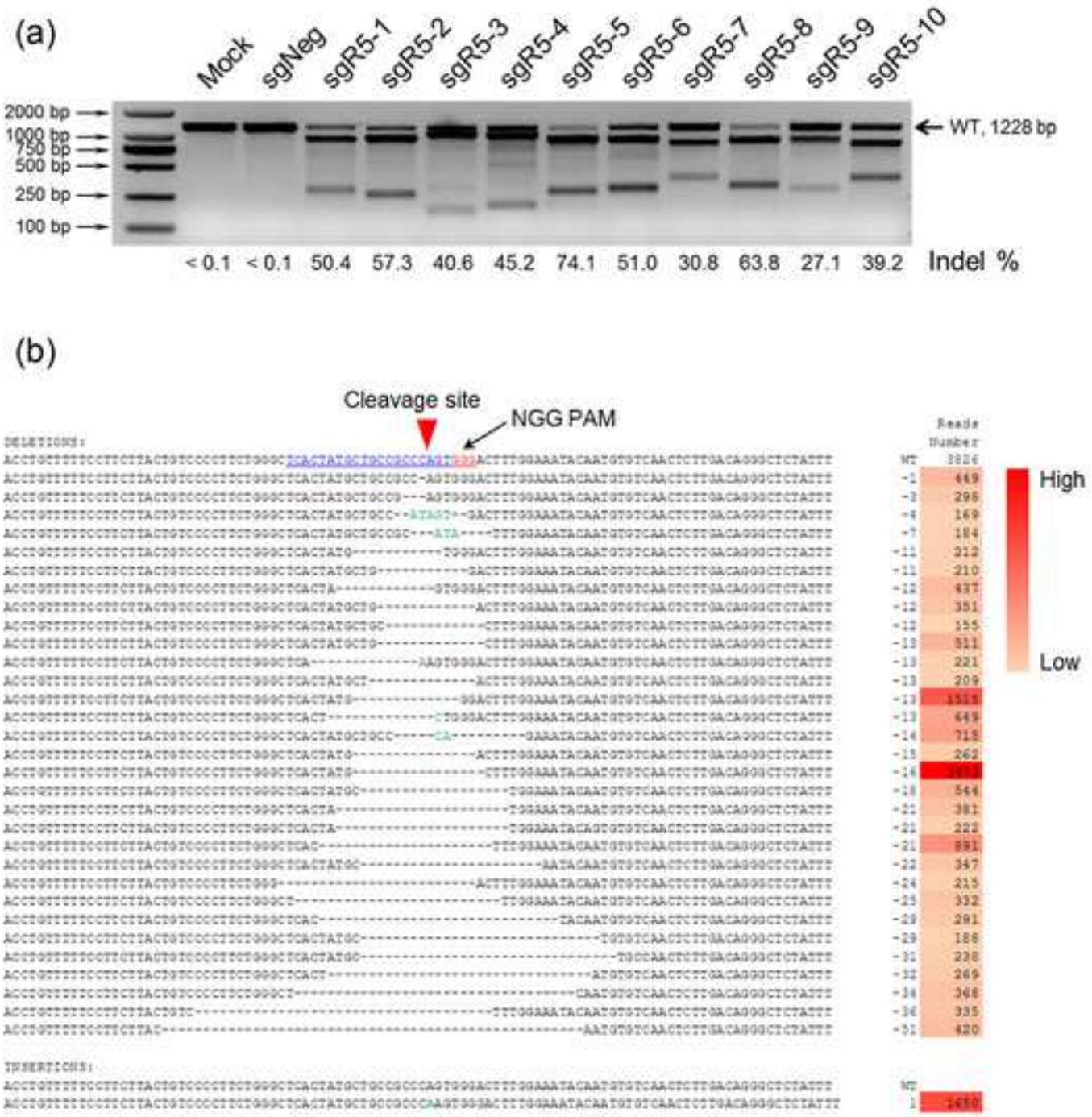


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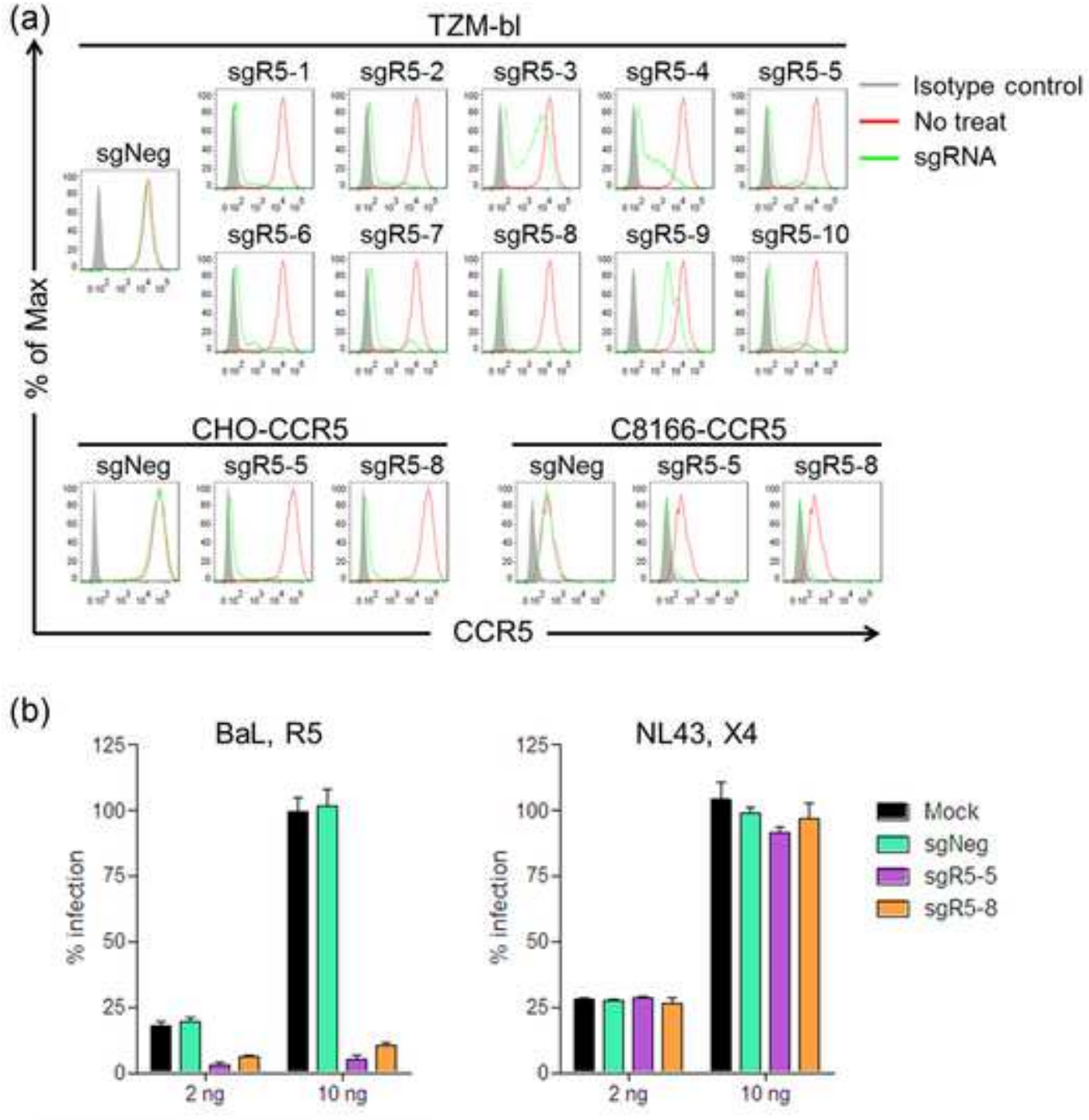


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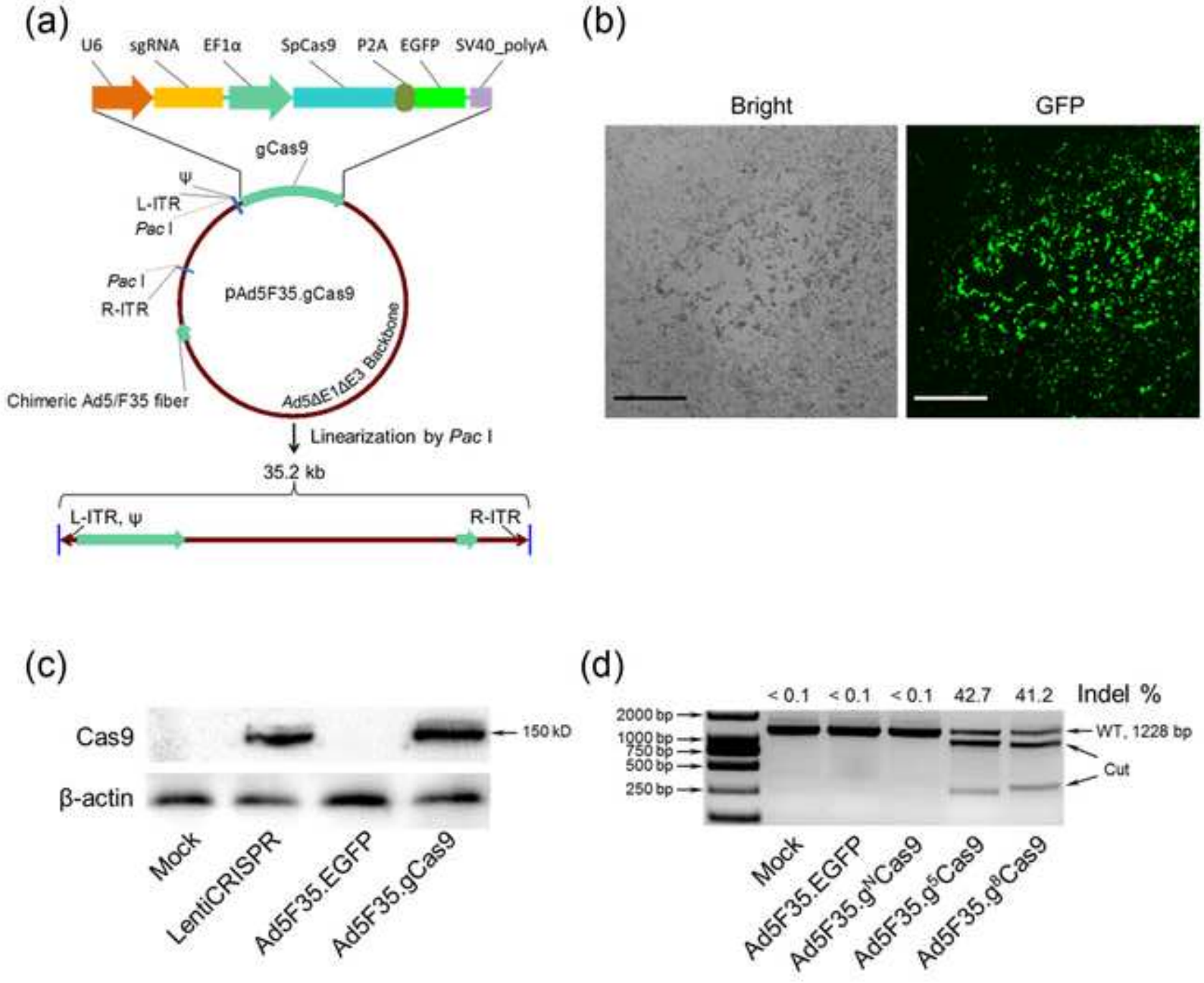


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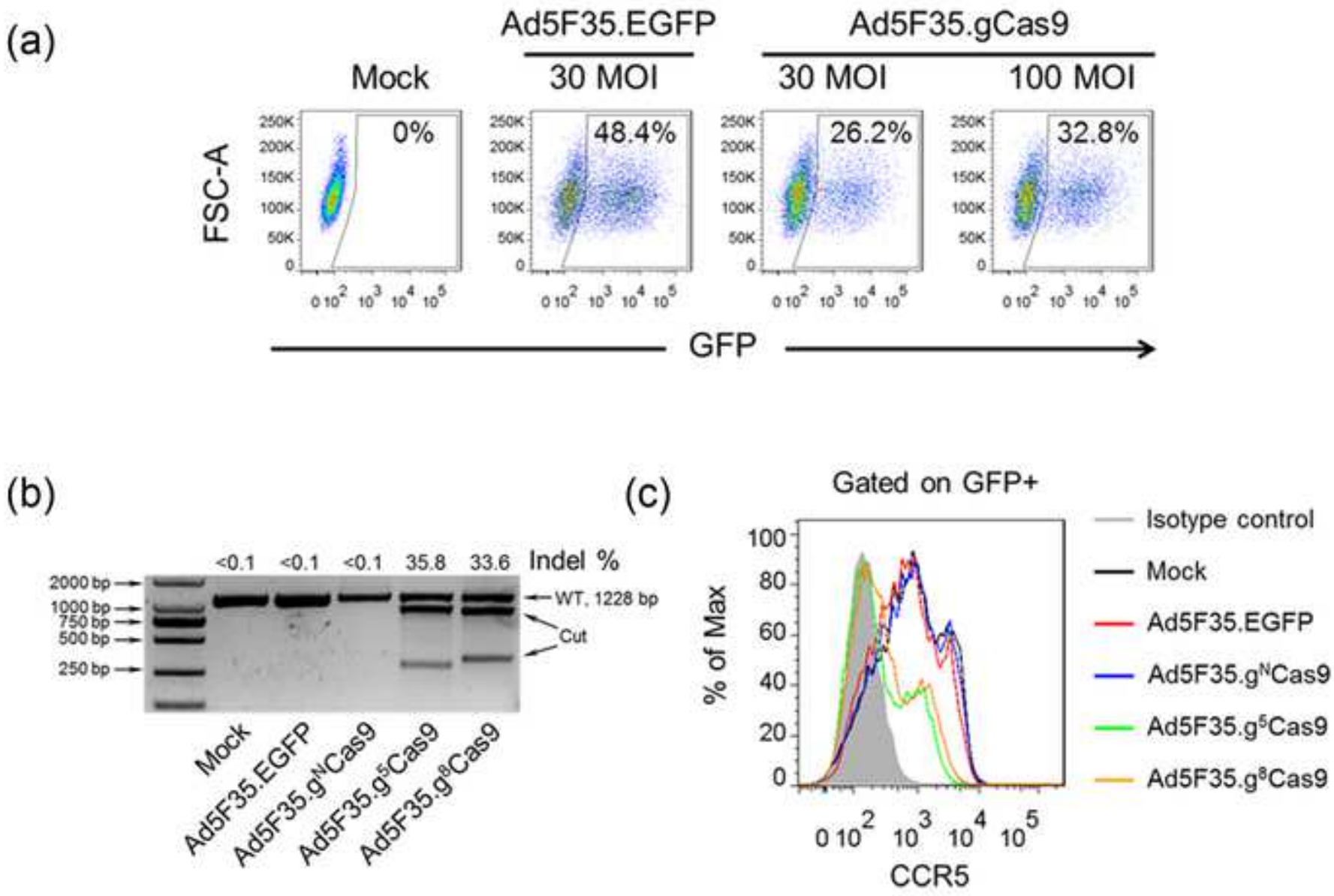
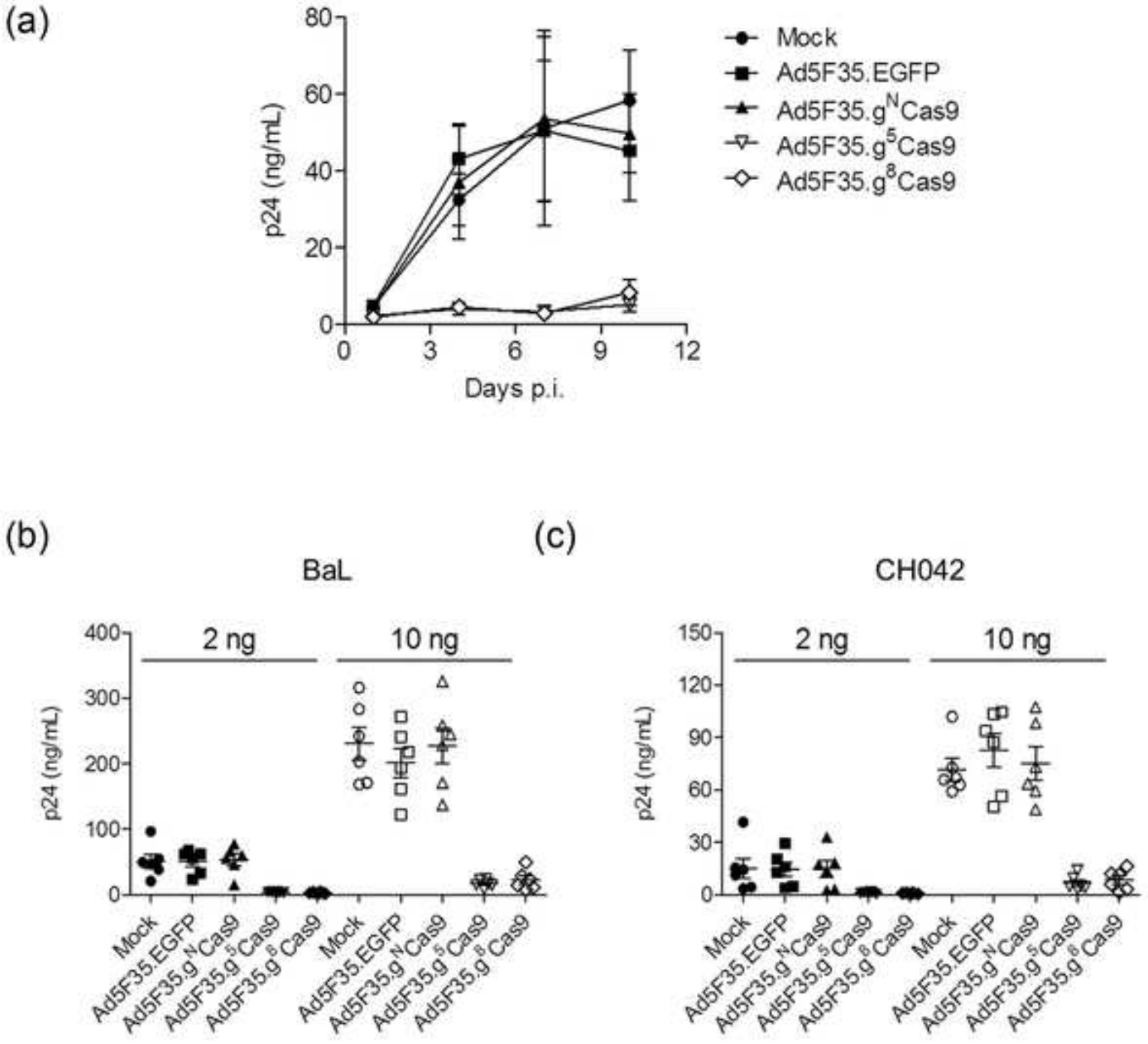


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