

Genetically engineered cell lines for α 1-antitrypsin expression

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Abstract

Objectives To establish genetically modified cell lines that can produce functional α 1-antitrypsin (AAT), by CRISPR/Cas9-assisted homologous recombination.

Results α 1-Antitrypsin deficiency (AATD) is a monogenic heritable disease that often results in lungs and liver damage. Current augmentation therapy is expensive and in short of supply. To develop a safer and more effective therapeutic strategy for AATD, we integrated the *AAT* gene (*SERPINA1*, NG_008290.1) into the AAVS1 locus of human cell line HEK293T and assessed the safety and efficacy of CRISPR/Cas9 on producing potential therapeutic cell lines. Cell clones obtained had the *AAT* gene integrated at the AAVS1 locus and secreted approx. 0.04 g/l recombinant AAT

into the medium. Moreover, the secreted AAT showed an inhibitory activity that is comparable to plasma AAT. **Conclusions** CRISPR/Cas9-mediated engineering of human cells is a promising alternative for generating isogenic cell lines with consistent AAT production. This work sheds new light on the generation of therapeutic liver stem cells for AATD.

Keywords AAVS1 locus · α 1-Antitrypsin deficiency · CRISPR/Cas9 · Homologous recombination

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Introduction

α 1-Antitrypsin deficiency (AATD) is one of the most common genetic disorders worldwide (de Serres and Blanco 2012). Patients with AATD suffer from early onset of pulmonary emphysema and have significantly higher risk of developing chronic obstructive pulmonary disease (Stoller and Aboussouan 2005). The current augmentation therapy, using intravenous administration of a pasteurized pooled human plasma AAT product to increase AAT levels in AATD individuals, has two major obstacles: source limitation and risk for emerging viruses (Chiuchiolo et al. 2013). Therefore, it is crucial to develop new therapies for AATD.

Chinese hamster ovary cells (CHO) are commonly used for biotherapeutic protein production. Despite recombinant AAT in CHO showing nearly equivalent

inhibitory activity in enzyme assays and serum half-lives, all sialylated glycans were in sharp contrast to those of natural AAT (Lee et al. 2013). Glycosylation of AAT plays crucial role in protein folding, therapeutic efficacy, in vivo half-lives and immunogenicity. Usually, glycans of recombinant protein in human cells are all human in structure and may reduce potential immunogenicity (Blanchard et al. 2011). For example, recombinant AAT in PER.C6 contains a human-type glycosylation pattern and has similar sialylation levels as plasma-derived AAT (Wang et al. 2013).

In this study, we targeted the *AAT* gene in AAVS1 ‘safe harbor’ of human HEK293T cells to elucidate whether CRISPR/Cas9-assisted homologous recombination could generate isogenic cell lines with consistent bioactive AAT production. The cell lines we obtained had the *AAT* gene inserted at the AAVS1 locus and showed constant AAT expression. As revealed by the elastase inhibitory assay, the produced AAT were biologically active.

Materials and methods

Chemicals were obtained from Sigma-Aldrich unless stated otherwise. The codon optimized *Cas9* and sgRNA expression vector (Genome-CRISPR human AAVS1 safe harbor gene knock-in kit, SH-AVS-K002) and knock-in ORF donor for *AAT* (SERPINA1, DC-F0215-SH01) were purchased from GeneCopoeia. The target site of sgRNA is GGGGCCAC-TAGGGACAGGAT, followed by the trinucleotide (5'-NGG-3') protospacer adjacent motif, which is recognized by the *Cas9* and essential for cleavage.

Cell culture, transfection, and stable cell line construction

HEK293T cells (ATCC CRL-3216) were grown in DMEM medium supplemented with 10% (v/v) FBS and incubated at 37 °C and 5% (v/v) CO₂. When grown to 80–90% confluency, cells were transfected with 2 µg DNA using Lipo2000 reagent (Gibco) and incubated in Opti-MEM reduced serum medium (Gibco). The donor plasmid contains a marker for puromycin resistance, which linked with *copGFP* by T2A. Cells that had integrated the transgene will be puromycin-resistant. Control cells were transfected with only the donor construct without CRISPR

plasmid. After 2 weeks of selection under puromycin dihydrochloride (2 µg/ml), a limiting dilution step was followed using the stable cell pools.

Quantitative real time PCR (qRT-PCR) for copy number analysis

For relative determination of copy number of transgene, qRT-PCR was performed using SYBR Premier Dimer Eraser (TaKaRa, Japan) with LightCycler480 (Roche) according to manufacturer’s instructions. Reaction mixtures contained SYBR Green QPCR master mix, 200 nM of forward and reverse primers, and 100 ng genomic DNA. The primers of transgene *AAT* and reference gene *GAPDH* are shown in Supplementary Table 1. All primers were validated empirically by melting curve analysis and agarose gel electrophoresis. Standard curves generated with two-fold serial dilutions of F0215-AAT plasmid that mixed with 100 ng genomic DNA of HEK293T cells over six grades showed a good linearity ($r^2 > 0.99$) and acceptable amplification efficiencies between 90 and 110%. Using a delta–delta threshold cycle method, relative copy number was estimated with respect to that of standard curves.

Western blot analysis

Native AAT purified from human plasma was purchased from Sigma-Aldrich. The recombinant AAT from the supernatant was collected after cells were removed. Protein samples (20 µg per lane) were denatured and separated by SDS-PAGE on a 12% gel and then transferred to a PVDF membrane in transferring buffer [0.025 M Tris, 0.19 M glycine, and 20% (v/v) methanol]. The membrane was treated with PBST/BSA [PBS, 0.1% (v/v) Tween 20, 1% (w/v) BSA] for 2 h to block the sites. The membrane was incubated overnight at 4 °C with a primary antibody and then incubated for 1 h with a secondary antibody. The bound antibody was detected using enhanced chemiluminescence detection reagents (Pierce) according to the manufacturer’s instructions. The band intensities were quantified with Kodak Image Station 4000 MM Pro (Kodak, Tokyo, Japan). Rabbit anti-human AAT polyclonal antibody (ab107773) and goat-anti-rabbit antibody was purchased from Abcam (Abcam, Cambridge, UK).

Quantification of AAT

ELISA was used for quantitative determination of secreted AAT levels in the medium using human AAT ELISA Quantitation Kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.). α -1 Antitrypsin potency was measured using a standard method in which AAT was incubated for 20 min with a stoichiometric excess of elastase (Sigma), then remaining elastase activity was quantified based on the method of Feinstein (Feinstein et al. 1973), measures the hydrolysis of Suc-Ala-Ala-Ala-pNa (Sigma) monitored at 405 nm.

Southern blotting

Southern blotting was carried out using Digoxin hybridization detection kits II (Roche). A *GFP* probe was generated by PCR using digoxin labeled dUTP mixture in PCR DIG Probe Synthesis Kit (Roche), with the entire transfection plasmid (F0215-AAT) as template. The primer sequences were shown in Supplementary Table 1. Genomic DNA (10 μ g) was digested with excess *Hind*III restriction enzyme. The digested genomic DNA was separated on 0.7% agarose gel and transferred to nylon membranes using conventional protocols. Linearized F0215-AAT plasmid digested with *Hind*III was used as a positive control. The membrane was hybridized with probe overnight at 37 °C at high stringency, followed by incubation with Anti-Dig-AP and CSPD, and exposure to X-ray film for 6 h.

Statistical analyses

To determine the statistical significance, we performed one-way ANOVAs using Dunnett's *t* test with SPSS 16.0 statistics software (SPSS Corp., Chicago, IL, USA). The difference was considered significant if $P < 0.05$.

Results and discussion

AATD is regarded as a predisposition to develop a number of diseases in both children and adults (Blanco et al. 2004). Development of new therapies using gene-modified hepatic stem cells may present a promising alternative for AATD treatment. The major challenge is to precisely modify the gene of interest

with high efficiency and safety (Wurm 2004). The adeno-associated virus site 1 (AAVS1) locus is a known 'safe harbor' in human genome, which circumvents gene silencing and insertional mutagenesis and provides persistent gene expression during cell expansion (Smith et al. 2008). No adverse effects were observed in transgenic mice and in human iPSCs when the AAVS1 locus is targeted (DeKolver et al. 2010; Henckaerts et al. 2009). Therefore, we efficiently integrated the *AAT* gene at the AAVS1 loci in HEK293T cells using CRISPR/Cas9 assisted homologous recombination (HR).

Transient expression of AAT in HEK293T cells

After transfection, the puromycin-resistant cells showed green and red fluorescence in R (*RFP*) and RA (*RFP* + CRISPR/Cas9) group, and green fluorescence in F (F0215-AAT) and FA (F0215-AAT + -CRISPR/Cas9) group (Fig. 1a). The secretion of AAT protein was further confirmed by ELISA and western blot; both showed higher AAT levels in 96 h medium than in 48 h medium. There was no difference in transient AAT expression between F and FA group (Fig. 1b, c), similar with the SDS-PAGE result (Supplementary Fig. 1).

AAT titers established by ELISA may not be accurate (Hansen et al. 2016), probably due to differences in glycosylation. Western blotting of the recombinant AAT showed a double band (Fig. 1c), which may also due to differences in glycosylation. Trace amounts of AAT in the 293T empty control may be expression of endogenous *AAT* gene in 293T cell (Fig. 1c).

Selection of cell clones with AAT integrated at the AAVS1 site

Junction PCR using primers spanning the intended junction site was employed to screen for targeted homologous recombination (Fig. 2). Cell clones that were positive by both 5'-junction PCR and 3'-junction PCR were selected. 15 clones were established with a mean positive rate of 28% (Table 1).

Identification of positive cell clones

As shown by the elastase inhibition assay, the recombinant AAT secreted by the positive cell clones

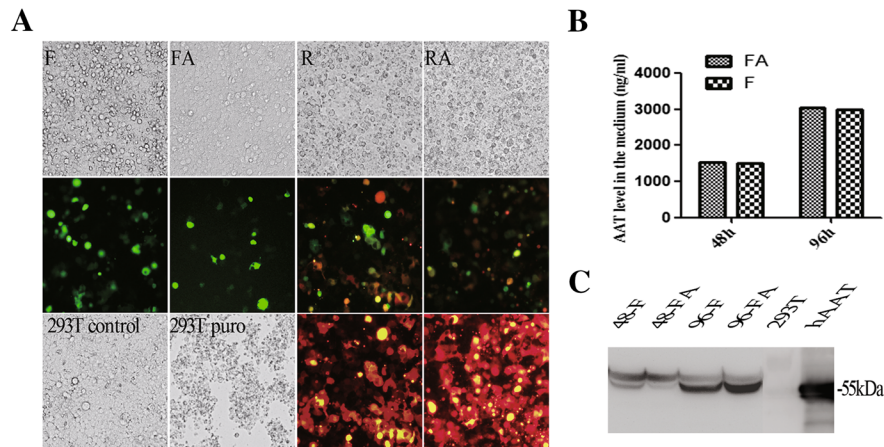


Fig. 1 Fluorescence microscopy assay (a) and expression level of AAT by ELISA (b) and Western blot (c) in puromycin-resistant HEK293T cells after transfection. *F* F0215-AAT plasmid, *FA* F0215-AAT plasmid + AAVS1 targeted

CRISPR/Cas9 plasmid, *R* RFP plasmid, *RA* RFP plasmid + AAVS1 targeted CRISPR/Cas9 plasmid, *293T puro* 293T cell with puromycin, *293T control* 293T cell with mock

PPP1R12C gene



Fig. 2 The diagram of knock-in verification with transgene AAT integrated at the human AAVS1 Locus in PPP1R12C gene. *F* forward primer, *R* reverse primer

had high biological activity (Fig. 3a). The clone B9, D11 and C10 showed significantly higher AAT levels (~ 0.04 g/l, $P < 0.05$). Although the ELISA result showed a lower AAT level than that of elastase inhibition assay for all clones (Fig. 3b) this may due to differences in glycosylation.

Copy number of AAT range from 0.9 to 14.8 in different clones (Fig. 3c), suggesting that, besides homologous recombination, random integration had also occurred. There may be an off-target phenomenon of CRISPR. There is evidence that cells with 2–5 copies of the transgene have the highest expression level of recombinant protein (Ross et al. 2012). Similarly, we found 2–4 copies of AAT gene in the top 3 (B9, D11 and C10) clones (Fig. 3c).

To identify the gene insertion at the AAVS1 locus, Southern blot (SB) assay was carried out in clone A8, B9, C10, D11 and 293T cell, whereas linear F0215-AAT (10077 bp) was used as positive control. When the AAT transgene was integrated at the AAVS1 locus by HR, the DNA fragments digested with *Hind*III, that detected by *GFP* probe, was 6146 bp in length. It was detected in these clones A8, B9, C10, and D11 except the 293T control (Fig. 4). Both junction PCR and Southern blot results demonstrated that at least one copy of AAT gene was inserted at the AAVS1 locus.

Table 1 The number of positive cell clones with AAT transgene integrated at the human AAVS1 safe harbor site

Batch	No. clone	No. GFP+ clone	GFP+ (%)	No. tAAT+ clone	tAAT+ (%)
1	29	12	41.8	3	25.0 ^a
2	27	14	52.4	2	14.3 ^a
3	39	22	56.4	10	45.5 ^b
Total	95	48	50.2	15	28.2

^{a, b} Values with different alphabets within columns are significantly different from each other ($P < 0.05$)

tAAT+ cell clone with AAT transgene integrated at the human AAVS1 safe harbor site

Fig. 3 Detection of the positive clones, **a** AAT potency assay, **b** ELISA and **c** gene copy number by Q-PCR

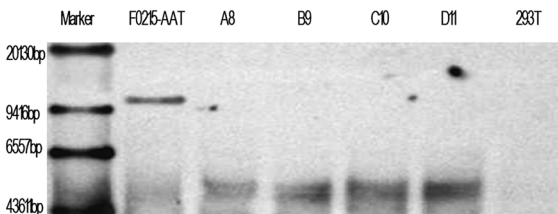
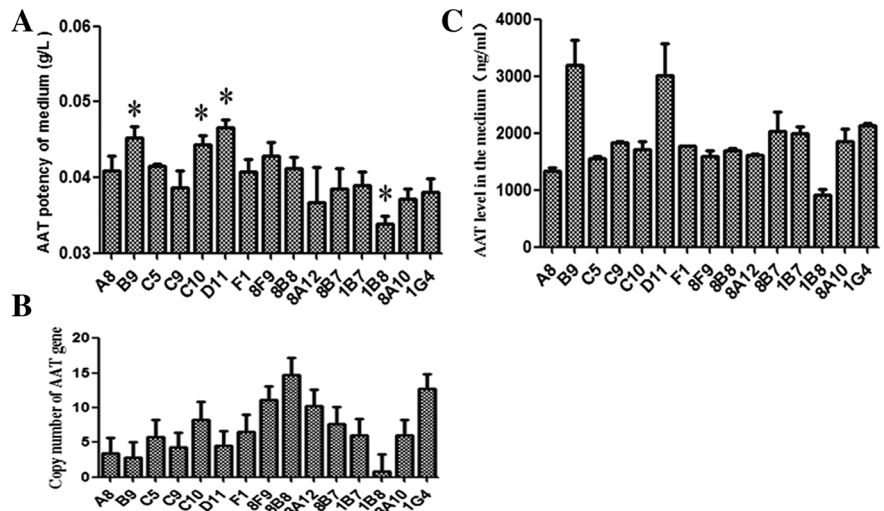


Fig. 4 Detection of the transgene at the AAVS1 locus by Southern blot

The expression feature of transgene cell clones

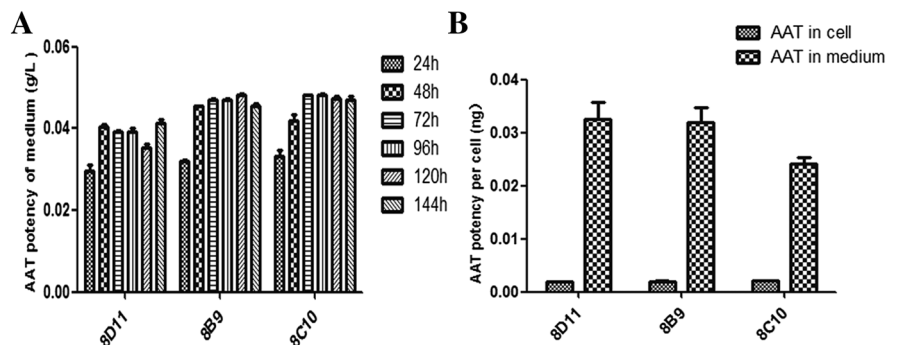
Three top clones were chosen for detection of AAT levels at different times. The medium was collected every 24 h when cells reached 90% confluence. As revealed by the elastase inhibition assay, AAT levels increased significantly from 24–48 h; whereas from 48–120 h, the levels of AAT were stable (Fig. 5a). After 120 h, expression of AAT and the attachment

ability of the cells decreased. This may result from contact inhibition.

The deficiency allele of AAT results in abnormal protein folding in endoplasmic reticulum, protein polymerization and intracellular retention. The secretion of the recombinant AAT was detected by elastase inhibition assay, comparing between AAT levels in cells and in the medium. Total proteins were extracted from cells of a known concentration. The AAT level in cells was significantly lower than that in the medium (Fig. 5b) suggesting that the active AAT was mainly secreted into the medium. However, there may be inactive AAT, which was intracellularly located.

Random integration of the introduced gene leads to reduced AAT production over time in generated cell lines (Wurm 2004). The AAVS1 locus has been shown to permit sustained and robust expression of integrated gene through extended time (Smith et al. 2008). After 50 passages, the cell clones still showed similar AAT expression, which were demonstrated as cell lines with

Fig. 5 The AAT potency assay of recombinant AAT at different time point (a) and secretion ability of the Top3 positive clones (b)



consistent protein production. Taken together, these results provide evidence that site specific integration by CRISPR/Cas9 assisted HR is likely to generate isogenic cell lines with consistent protein production.

Conclusion

The *AAT* gene was efficiently targeted into the AAVS1 loci in HEK293T cells which produced bioactive recombinant AAT proteins. Integrating the *AAT* gene into human cells by CRISPR/Cas9-assisted homologous recombination is thus a promising strategy for generating therapeutic cell lines for AATD. This study has paved the way for further establishment of gene-corrected liver stem cells for curative treatment of AATD.

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Supporting information Supplementary Table 1—Primer sequences.

Supplementary Figure 1—SDS-PAGE of AAT in puromycin-resistant HEK293T cells after transfection.

Compliance with ethical standards

Conflicts of interest The authors have no conflict of interest to declare, and no competing financial interests exist.

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