Transient to stable non-viral gene therapy strategies for the correction of cystic fibrosis

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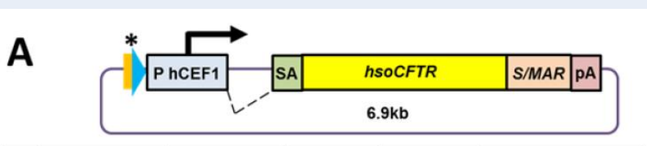
Cystic fibrosis is one of the most common inherited lethal diseases, caused by mutations in the *CFTR* gene. Small-molecule therapies have revolutionised patient outcomes but are lifelong interventions and cannot correct all *CFTR* mutation variants.

We have demonstrated effective non-viral gene delivery to mice using peptide-nanocomplexes composed of plasmid (p)DNA and GET peptides, which bind and transduce cell membranes. Our formulations utilise endosomal-escaping strategies to deliver gene correction/augmentation strategies, presenting a 'genetic cure' which includes treatment of CF patients unaffected by current state-of-the-art therapies.

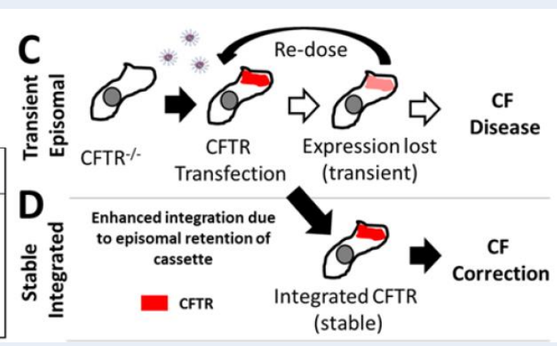
We have confirmed our system's efficacy with fluorescent-protein encoding *ZsGreen1* and will confirm CF correction in patient-derived lung cells with *CFTR* transgene pDNAs. Presently, we are comparing strategies: HDR (homology driven repair) and HITI (homology-independent targeted integration) via CRISPR. We are also exploring directed integration with Rep-mediated (exploiting viral mechanisms targeting P5IEE to the AAVS1 locus) systems and *Sleeping beauty* transposase.

1. Gene delivery to correct CF phenotype.

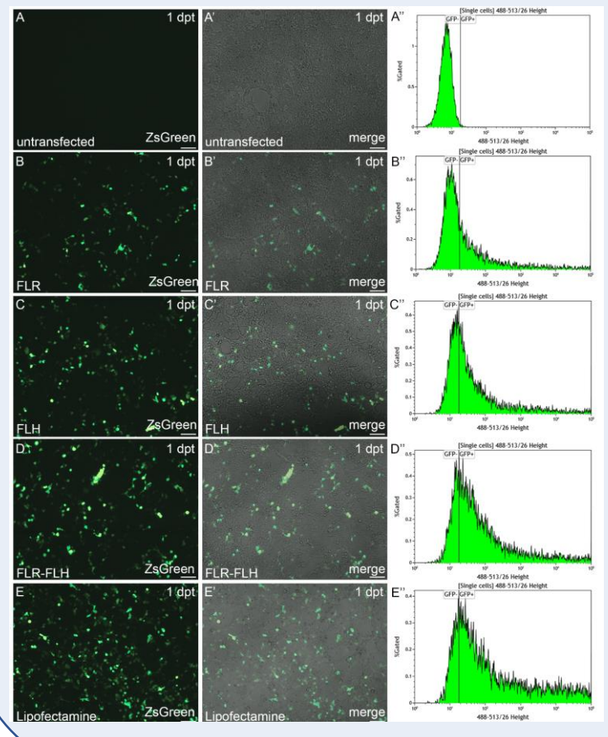
CF patients lack functional CFTR protein, cause by mutations in the CFTR gene – delivery of full length CFTR permanently to patient genomes would restore functional CFTR protein, alleviating disease symptoms.



B	Integration Strategy	Integration Element (*)	Co-Factors	Integration Site	Promoter for integation expresion
1	HDR	Homologies	Cas/gRNA, ZFNs,	*AAVS1, CFTR*	Transgenic or endogenous
2	HITI (NHEJ)	Inverted gRNA	Cas/gRNA	*AAVS1, CFTR*	Transgenic or endogenous
3	SB	SB ITRs	SB100X	Random	Transgenic
4	Rep	p51EE	Rep68/78	*AAVS1*	Transgenic

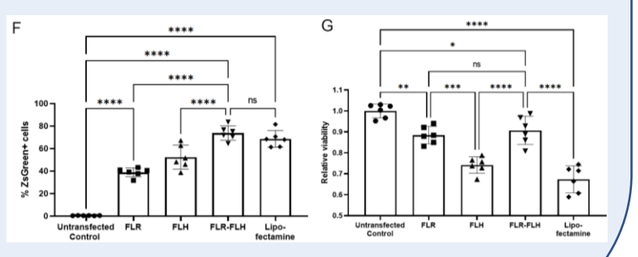


2. GET gene delivery is comparable to lipofectamine with higher cell viability

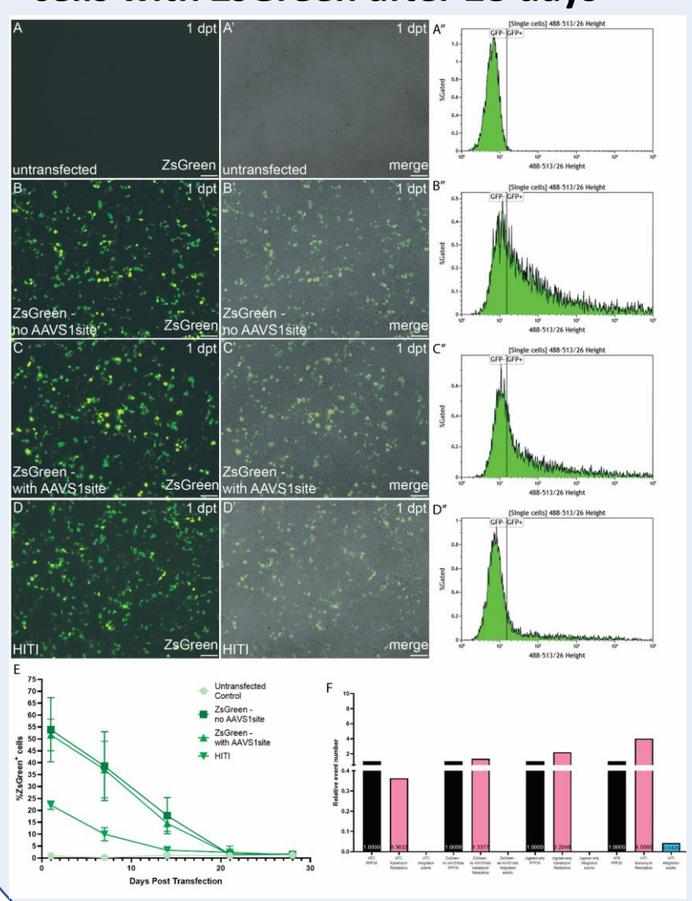


Delivery of ZsGreen plasmid by either of the GET peptides (FLR or FLH, **B-C''**) alone is less effective than combined (GET/FLR-FLH) peptides (**D-D''**) or lipofectamine delivery (**E-E''**).

Delivery by GET or lipofectamine yields a ~60% transfection efficiency, while FLR or FLH alone yield <60% (**F**). Importantly, cell viability following GET delivery is significantly higher than lipofectamine (**G**).



3. GET-mediated HITI delivery permanently labels HEK293T cells with ZsGreen after 28 days



Transfection of HITI (**D-D''**) yields lower numbers of ZsGreen+ cells than ZsGreen plasmids transfected alone (unmodified ZsGreen **B-B''**, and Zsgreen modified to enable HITI integration **C-C''**).

By 21-28 days, ZsGreen expression is comparable in all transfected cell lines, indicating a greater level of longevity in HITI transfection (**E**).

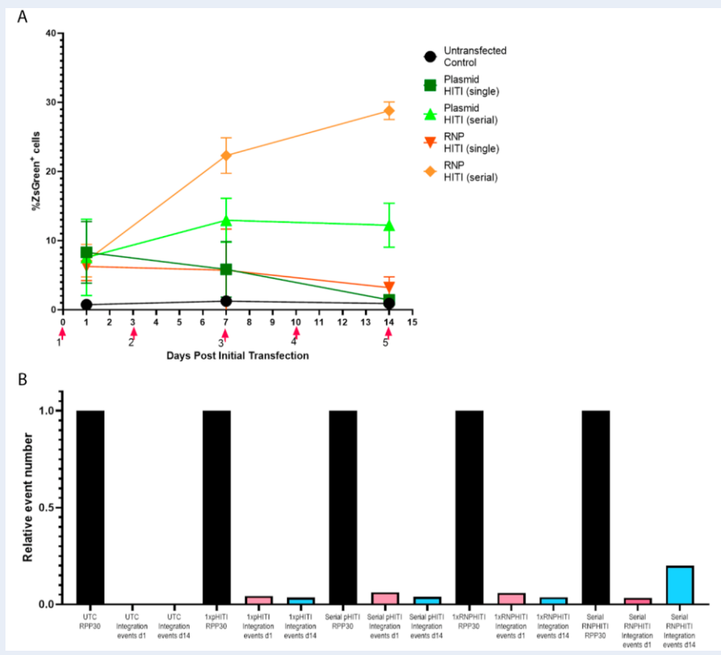
Importantly, integration events, detected by ddPCR, indicate ~4% of HITI-transfected cells display genetic integration (**F**). This shows that HITI-mediated CRIPSR integration permits stable integration over long time periods.

4. Serial GET-mediated HITI delivery enhances genomic integration

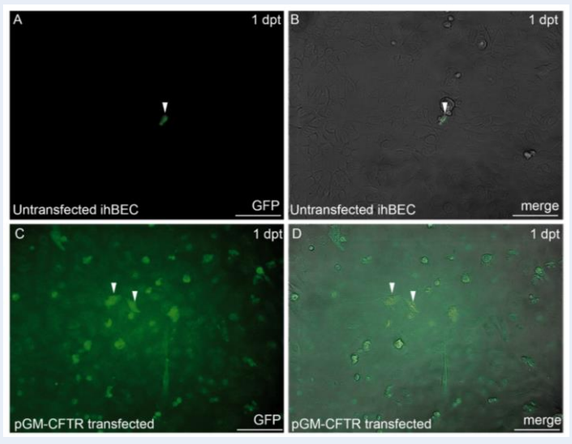
Preformation of RNP complexes for CRISPR-based experiments have been suggested to increase CRISPR efficacy. We performed serial delivery of HITI, both via delivery of constituent plasmids or by forming RNP complexes prior to transfection.

We transfected every 3-4 days (**A, red arrows**) and observed an increase in ZsGreen expression in serially-transfected cells (**A**).

Analysis via ddPCR showed increased integration rate in cells which were serially-transfected by RNP complexes, but not in cells transfected by plasmids (**B**), indicating precomplexation increases integration efficiency

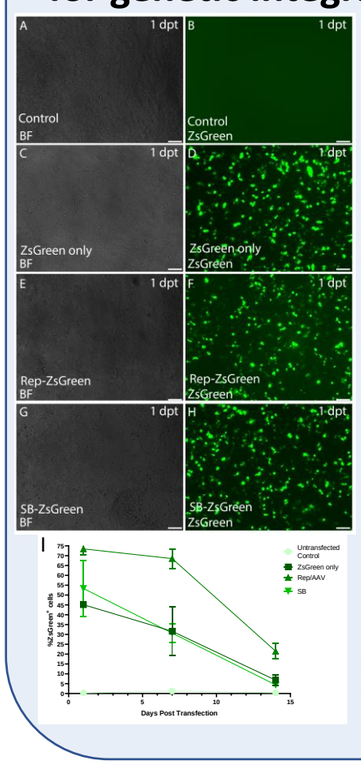


5. Episomal CFTR expression in ihBEC airway cells



Untransfected ihBECs display limited CFTR protein detectable by immunohistochemistry (**A-B**), while ihBECs transfected with pGM-CFTR display increased levels of CFTR expression 1 day post transfection

6. SB and Rep/AAV mediated gene delivery may be suitable for genetic integration



Rep/AAV genetic integration preferentially targets the AAVS1 "safe harbour" locus, while Sleeping beauty transposase (SB) can deliver to any part of the genome.

Preliminary analysis indicates Rep/AAV-mediated (**E, F, I**) ZsGreen delivery confers greater longevity of expression than SB (**G-I**).

Conclusions

- GET peptides are viable for non-viral gene delivery of pDNA to human cells, and are less cytotoxic than lipofectamine.
- HITI can be used to deliver full length gene coding sequences to specific genetic loci and allow them to integrate permanently, enhanced by serial delivery.
- Delivery of full length CFTR to lung cells demonstrates specific expression episomally

Future directions

- Deliver full-length CFTR to patient-derived airway epithelial cells, using serial delivery to enhance permanent integration.

References

1. Suzuki, K., Tsunekawa, Y., Hernandez-Benitez, R., Wu, J., Zhu, J., Kim, E.J., Hatanaka, F., Yamamoto, M., Araoka, T., Li, Z. and Kurita, M., 2016. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature*, *540*(7631), pp.144-149.
2. Osman, G., Rodriguez, J., Chan, S.Y., Chisholm, J., Duncan, G., Kim, N., Tatler, A.L., Shakesheff, K.M., Hanes, J., Suk, J.S. and Dixon, J.E., 2018. PEGylated enhanced cell penetrating peptide nanoparticles for lung gene therapy. *Journal of controlled release*, *285*, pp.35-45.