The immunosuppressive TTAGGG motif improves homology-directed insertion of DNA sequences in human induced mesenchymal and pluripotent stem cells

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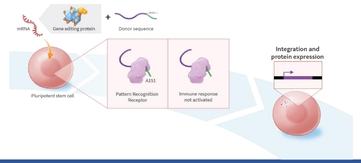
Abstract

Synthetic oligodeoxynucleotides (ODNs) have been used as donor templates in gene-editing applications to insert transgenic sequences into defined genomic loci, albeit with low efficiency. To improve integration, gene-editing proteins can be co-expressed to create a double-strand break at the target locus. Recognition of double stranded donor templates (dsODN's) by pattern recognition receptors activates signaling cascades resulting in the production of cytokines, including type I interferons such as IFIT1-3 and IFN-β. This immune response can lead to cell cycle arrest, differentiation, and apoptosis, contributing to low insertion efficiency observed in human cells. Here, we explore the effect of the TTAGGG motif on dsODN -related activation of a pro-inflammatory response in human cells, and transgene insertion efficiency. We incorporated the TTAGGG motif either at the 5' and 3' ends of dsODN, or co-delivered it separately as a short single-stranded ODN to improve cell health and editing efficiency of human primary, iPS and iMSCs cells.



Introduction

Synthetic oligo nucleotides are of interest for many cell engineering based therapeutics, including the generation of allogeneic NK and T cells that are editing to express stealthing proteins, cytokines, and chimeric antigen receptors (CARs) for the treatment of a variety of cancers. Many challenges still remain in the generation of such stable cell lines, for example, due to the toxicity of the ODNs. It has been shown in human immune cells that co-delivery of a short ODN comprising the immunosuppressive motif, TTAGGG, which is found in mammalian telomeric DNA, inhibits the activation of the damage-associate molecular pattern (DAMP) pathway in response to cytosolic DNA. This ODN competitively binds to inflammasomes, and reduces the secretion of proinflammatory cytokines. The use of this motif has not previously been explored to understand its role in enhancing gene editing.

Generation of the TTAGGG oligo and repair template

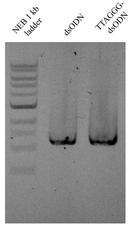
The TTAGGG motif found in telomeric DNA was incorporated onto the ends of a double stranded donor template (dsODN) or co-electroporated as a short oligo fragment in human primary, iMSCs and iPS cells. The motif is recognized by, and competitively binds to Pattern Recognition receptors in the cytoplasm of the cells. The donor templates were amplified using the Primer pairs (Fig 1) and PCR protocol shown in Table 1 below. Both templates with and without the TTAGGG motif were amplified using the same conditions.

A) **Telomeric DNA Repeat motif:** TTAGGGTTAGGGTTAGGGTTAGGG

B) **Primer pairs:**
AAVS1 – Forward primer: CCGAGCTGGGACCACCTTATATTCCCAGG
AAVS1 – Reverse primer: AGGAGGTGGGGGTTAGACCCAATATCAGG

TTAGGG-AAVS1 – Forward primer:
T*T*A*G*G*G*T*T*A*G*G*G*T*T*A*G*G*G*T*T*A*G*G*GCCGAGCTGGG
ACCACCTTATATTCCCAGG

TTTAGGG-AAVS1 – Reverse primer:
T*T*A*G*G*G*T*T*A*G*G*G*T*T*A*G*G*G*T*T*A*G*G*GAGGAGGTGGG
GGTTAGACCCAATATCAGG



Step	Cycle	Condition	
		Temperature (C)	Time
1	-	95	5 minutes
2	33	98	20 seconds
		68	1 minute
3	-	68	2 minutes
		4	-

Figure 1 & Table 1) *The PCR amplification and primers used to generate a dsODN repair template with and without the TTAGGG motif at both the 5' and 3' ends. The primary repair template was designed to target the AAVS1 site with a right and left homology arm. A GFP tag was incorporated under a pJeT promoter. The repair template included an Sfo1 restriction site and synthetic Poly-A sequence. The donor sequence was 1.2 kb long. The TTAGGG motif included a phosphorylated backbone that was synthesized by IDT Technologies, and is indicated by a *.*

GFP expression and cell viability

Human fibroblast cells, induced pluripotent stem cells and induced mesenchymal stem cells were all electroporated with 300 ng of a donor template, and 1 ug of gene editing mRNA, UltraSlice, targeting the AAVS1 loci. The TTAGGG motif was introduced into cells either via co-electroporation, or using the modified dsODN template that contained the TTAGGG motif on the 5' and 3' end of the donor template (Fig. 2 – 4). Cells were electroporated using the Invitrogen Neon Transfection system.

Fibroblast cells

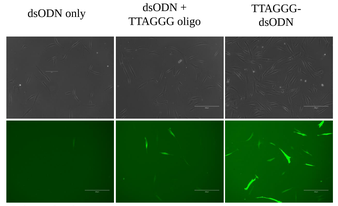


Figure 2) *Fibroblasts expressed GFP 24 hours after electroporation, with the inclusion of the UltraSlice mRNA. There was increased cell count and GFP expression in human primary cells in the presence of the TTAGGG motif was present, either as the short oligo, or on the modified repair template.*

Induced Pluripotent Stem Cells

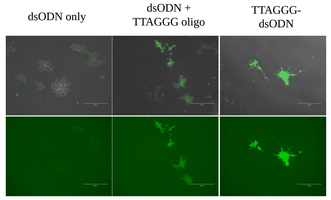


Figure 3) *iPSCs were electroporated with the mRNA UltraSlice gene editing protein targeting AAVS1. The TTAGGG motif was co-electroporated along with the dsODN repair template. Cells were kept in culture and showed GFP expression after 3 days. By Day 5, GFP tagged colonies had formed and cells continued to proliferate in all conditions.*

Induced Mesenchymal Stem Cells

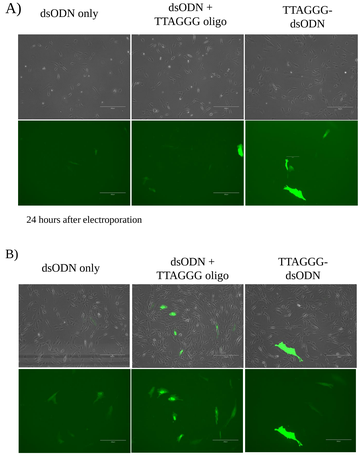


Figure 4) *iMSCs electroporated with the mRNA UltraSlice gene editing protein, expressed GFP 24 hours after (Fig 4A). There was increased intensity in the presence of the TTAGGG motif. Further, cell count and recovery were higher compared to the electroporation with the control template only. GFP tagged iMSCs continued to divide and proliferate 72 hours after electroporation (Fig. 4B).*

iPSCs and iMSCs characterization

The iMSCs and iPSCs were characterized by gene expression by RT – PCR (Fig. 6) and flow cytometry (Fig. 8) to asses the impact of the TTAGGG motif on cell state.

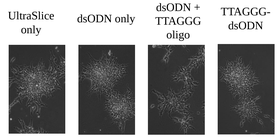


Figure 5) *iPS cells 1 week after electroporation had all formed colonies displaying the correct phenotype.* ***Figure 6)*** *RT PCR results showed that there was no statistically significant difference (P >0.05) in NANOG or SOX2 expression in the presence of the TTAGGG motif. RT PCR was performed on RNA harvested 24 hours after electroporation of the dsODN repair templates or TTAGG oligo only.*

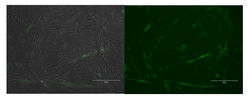


Figure 7) *iMSCs were maintained and passaged for 28 days after electroporation of the dsODN and TTAGGG oligo. GFP expression was maintained. GFP tagged cells had a viability of > 97% for all populations and maintained a similar expansion timeline compared to the control iMSCs.*

Markers	Expected results	Control iMSCs (%)	dsODN + oligo iMSCs (%)
CD34	Negative	8.0	0.0
CD44	Positive	99	100
CD45	Negative	6.0	10
CD73	Positive	99	100
CD90	Positive	98	99

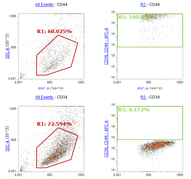


Figure 8) *iMSCs displayed the expected surface markers after electroporation with the TTAGGG motif present. The iMSCs were stained, fixed, and flowed on the AttuneNx for CD34, CD44, CD45, CD73, and CD90. Compared to a control population of iMSCs, the populations displayed the same flow results. Briefly, both populations were positive for CD44, CD73, and CD90. Cells were negative for CD34 and CD45. Figure 6B shows the spread of the population of iMSCs. The populations showed clear signs of surface marker expression if they were positive (CD44) or negative (CD34). The flow data indicates that the inclusion of the motif did not alter the surface markers, nor the viability of the cells after electroporation.*

Assessing the immune response to the TTAGGG motif

iPSCs and iMSCs were electroporated without the gene editing mRNA to study the effect of the TTAGGG motif on the immune response to the double stranded repair template during electroporation. Cells were harvested 24 hours after electroporation and an RNA extraction was performed using Zymo Research Quick-RNA Miniprep kits. The RT-PCR was performed on the QuantStudio 6 Flex by Life Technologies (Fig. 9). The presence of the motif reduced the upregulation of the IFIT pathway in iMSCs and iPSCs that was due to the dsODN.

A) iPSCs

	Negative	dsODN Only	Oligo only	dsODN + oligo	TTAGGG-dsODN
IFIT1	1.00	2.76	0.87	0.62	2.20
IFIT2	1.00	3.83	0.23	0.11	2.54
IFIT3	1.00	1.12	0.755	0.402	0.625
IFIT5	1.00	0.904	0.930	0.635	0.743
IL18	1.00	1.30	1.75	1.5	1.06
INF-Beta	1.00	1.41	0.763	0.534	1.45

B) iMSCs

	Negative	dsODN Only	dsODN + oligo	TTAGGG-dsODN
IFIT1	1.00	28.1	0.0590	16.8
IFIT2	1.00	6.32	1.26	4.36
IFIT3	1.00	29.4	1.96	11.0
IL18	1.00	0.414	1.27	0.820
IFN-Beta	1.00	1.58	1.52	0.848

Fig. 9) *iPSCs and iMSCs Gene Expression 24 hours after electroporation of the dsODN repair template, with and without the TTAGG motif. The presence of the motif downregulated the immune markers IFIT1-3 in both iMSCs and iPSCs, compared to the dsODN only control. This was most noticeable in the iMSCs where the dsODN had increased expression of IFIT1 and IFIT3 by 28.1 and 29.4 respectively. IL18 expression was decreased in iPSCs, but neither IL18 or IFN-beta seemed to be upregulated by the presence of the dsODN. All conditions were run with n = 3 samples. The RQ values were normalized to the GAPDH probe in the electroporation only, negative sample.*

Gene-editing efficiency

To assess the impact that the TTAGGG motif had on editing efficiency, iPS cells were extracted 1 week after electroporation and a T7E1 assay was performed (Fig. 10). DNA was extracted from iMS cells that had been maintained in culture for 28 days and a T7E1 was run (Fig. 11)

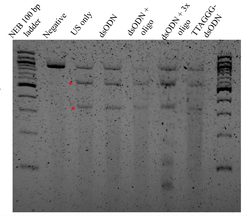
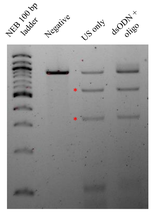


Figure 10) *iPSCs electroporated with the TTAGGG 200 ng and 600 ng of the oligo showed increased gene editing (44% and 50%) than the UltraSlice only and dsODN control (41% and 33%) indicating that the presence of this repeat sequence may improve gene editing.*

Lane	Band 1	Band 2	Band 3	Editing %
Negative	95	-	-	
US only	35	43	22	41
dsODN	45	35	20	33
dsODN + TTAGGG	30	51	18	44
dsODN + 3x TTAGGG	23	54	21	50
TTAGGG-dsODN	42	26	30	34

**Editing efficiency calculated based on NEB formula for T7E1 assay*



Lane	Band 1	Band 2	Band 3	Editing %
Negative	87	5	7	
US only	36	36	27	39
dsODN + TTAGGG	36	24	38	38

Figure 11) *iMSCs electroporated with the TTAGGG oligo showed similar signs of gene editing than the UltraSlice only control even after 4 passages. The NEB protocol for the T7E1 assay was followed. * indicates T7 cut sites.*

Conclusions

The telomeric repeat sequence TTAGGG motif can either be incorporated at the 5' and 3' ends of a repair template or co-electroporated along with a dsODN and gene editing mRNA in order to decrease the immune response of cells to the double stranded foreign DNA in the cytoplasm. This may lead to increased integration into the overall cell population by improving cell viability after electroporation. We demonstrate for the first time that this sequence can be used in gene editing of primary fibroblasts and iMSCs and iPSCs in order to improve the generation of stable, edited cell lines.

CR and MA are inventors on U.S. Pat. Nos. 8,497,124; 9,127,248; 9,399,761; 9,562,218; 9,695,401; 9,879,228; 9,969,983; 10,131,882; 10,201,599; 10,443,045; and others.