A new chapter on targeted gene insertion for X-CGD: do not skip the Intro(n)

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X-linked Chronic Granulomatous Disease (X-CGD) is a rare, devastating primary immunodeficiency disorder (PID), characterised by defective host defence against certain bacteria and fungi.[1] It is caused by mutations in the *CYBB* gene, encoding the gp91phox subunit of NADPH oxidase, an enzyme complex responsible for pathogen clearance in professional phagocytic cells. As for many monogenic disorders of the immune system, X-CGD is amenable to gene therapy through gene addition. More recently, scientists have also started to explore nuclease-mediated targeted gene insertion as a strategy to restore gp91phox expression in reprogrammed stem cells (iPSCs) [2-4] and Haematopoietic Stem and Progenitor cells (HSPC) [5] from CGD patients. In this current paper, Sweeny et al. [**ref 6**], show that nuclease-mediated insertion of a CYBB minigene into exon 2, but surprisingly not exon 1, can restore gp91phox protein expression in iPSCs -derived granulocytes from X-CGD patients (Figure 1). This study highlights the importance of intronic elements for the expression of the *CYBB* gene by the endogenous regulatory elements and cautions those developing strategies for genetic correction by targeted insertion of minigenes.

HSPC gene therapy by the use of viral vectors, has proven to be an effective therapeutic option for a series of PIDs. However, previous clinical trials for CGD, based on the use of gamma retroviral vectors, have in general failed to demonstrate significant long term engraftment of gene corrected cells in the absence of mutagenic proliferation of myeloid precursors.[7] The recent introduction of lentiviral vectors with myeloid regulatory elements may not only improve safety by obviating enhancer-mediated mutagenesis in HSPCs, but may also enhance gene expression towards more physiological levels. Clinical trials in US and Europe are currently underway. Alternatively, transposon-mediated integration of a BAC vector carrying the whole genomic region of the *CYBB* gene has been shown to rescue the CGD phenotype in granulocytes derived from iPSCs.[8] However, clinical application of BAC trans-genesis for the gene therapy of CGD or other disorders is challenging because of the size of payload required to deliver the necessary regulatory elements as well as the coding sequence.

DNA editing techniques have opened up new exciting avenues for gene therapy of genetic disorders. [9] Scientists can now use target directed nucleases to create a double strand break anywhere in the genome, and insert a new DNA sequence into the break by Homology Directed Repair (HDR). This approach has been used, for example, to insert minigenes and, among those, the *CYBB* minigene into the AAVS1 locus, a putative genomic safe harbour, also resistant to silencing. [2, 3, 5] Although in principle safe, this strategy is still not perfect, for there is only a partial rescue of NAPDH-oxidase function in fully differentiated neutrophils . Alternatively, to capture the advantages of natural gene regulatory mechanisms, scientists can use engineered nucleases to insert a functional minigene near the transcription start site of the disease-causing gene, also taking advantage of the increased cutting efficiency of the nuclease in this part of the gene structure.[10] The assumption here is that it will enable physiological expression of the protein of interest since transcription is driven by the gene's own regulatory sequences. Another even more precise approach would be to repair the pathogenic mutations *in situ*. For example, efficient targeted repair of the beta globin gene has been achieved in HSPCs by combining the use CRISPR/Cas9 system with AAV6-mediated delivery of a homologous donor template.[11] However, for the majority of genetic disorders where mutations can be spread across the whole gene, the wider application of this approach seems at present unlikely particularly for future commercial development.

The optimization of protocols to differentiate iPSCs into neutrophils [3] has given impetus to study new gene therapy approaches for CGD as well as to find ways to use those cells as potential source for therapeutic granulocyte infusion. In this paper, Sweeny et al., have compared different gene editing strategies for the correction of the X-CGD phenotype using iPSCs-derived granulocytes from CGD patients. Firstly, they accurately corrected the *CYBB* gene in iPSCs cells from patients bearing a 458T>G mutation or a 461(A) deletion in the exon 5. For this purpose they nucleofected TALEN or CRISPR/Cas9 expression plasmids targeting exon 5 together with a donor plasmid bearing the correct homologous sequence, and excisable selection markers. Clones that had undergone granulocytic differentiation exhibited almost normal levels of gp91phox expression and NADPH oxidase function. Remarkably, the team also detected normal expression levels of the CD13 marker

suggesting that gene repair had unexpectedly contributed to normal granulocytic differentiation in CGD cells.

They next devised a universal strategy to complement almost all gene mutations. For this purpose, they nucleofected TALENs or CRISPR/Cas9 plasmids targeting exon 1 or exon 2 of the CYBB locus together with a plasmid containing two versions of the CYBB mini gene (exon 1-13 or exon 2 -13). They showed that TALEN -mediated insertion of a complete CYBB minigene exon 1-13 at the transcription start site of the gene (Figure 1a), did not lead to gp91phox expression or NADPH oxidase activity. In contrast, CRISPR/Cas9 -mediated insertion of the CYBB exon 2-13 mini gene at the exon 2 site (Figure 1b) restored levels of gp91phox and of NADPH oxidase activity that were 64-100% and 68-100% of those obtained in control blood, respectively. Notably, expression of gp91phox was strictly myeloid-specific as the protein was only found in the CD13 positive population. The different outcomes of the two strategies suggest that an intact exon/intron 1 is key for normal endogenous expression of gp91phox. This should not come as too much of a surprise as it is widely recognised that intronic sequences can play a crucial role in normal gene expression. Intronic regions have also been used to boost gene expression or guide transcription in a tissue/stage specific manner. [12]

Although the authors did not investigate the mechanisms by which intron 1 controls expression of the CYBB gene, it is possible that the faulty correction arises from a lack of exon/intron splicing, the absence of an Exon/Junction complex signature, or the loss of enhancer elements that impairs expression of the CYBB minigene exon 1-13 from its own promoter. Promoter-proximal introns often contain recruitment sites for enhancers or silencers. Maintaining early introns, as recently done for targeted editing of the Wiskott Aldrich Syndrome gene[13], may therefore be a valid strategy for gene correction. As the authors rightly point out in the discussion, putative NF-kB binding sites are present in the first two introns of *CYBB*, suggesting that these could behave as enhancers. This is in line with the observation made by others that a minimal *CYBB* gene promoter is not sufficient to drive normal levels of expression of the protein in the context of lentiviral vectors. [14]

The use of selectable markers in the targeting constructs precludes evaluation of the efficiency of repair. Nevertheless, the message of this study remains strong and clear. A careful analysis of the different elements required for physiological levels of gene

expression is an essential step for emerging gene editing technologies as it will ultimately dictate the optimal strategy to achieve therapeutic levels of correction.

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Figure Legend

Figure 1. Overview of the gene targeting strategies devised by Sweeny et al.[6] to complement CYBB gene mutations in induced pluripotent stem cells (iPSCs) from patients with X-linked chronic granulomatous disease (X-CGD). Rescue of the Gp91phox protein expression in X-CGD iPSC-derived granulocytes by CRISPR/Cas9 -mediated insertion of the CYBB exon 2-13 mini gene at the exon 2 site A) but not by TALEN -mediated insertion of a complete CYBB minigene exon 1-13 at the exon 1 site B).