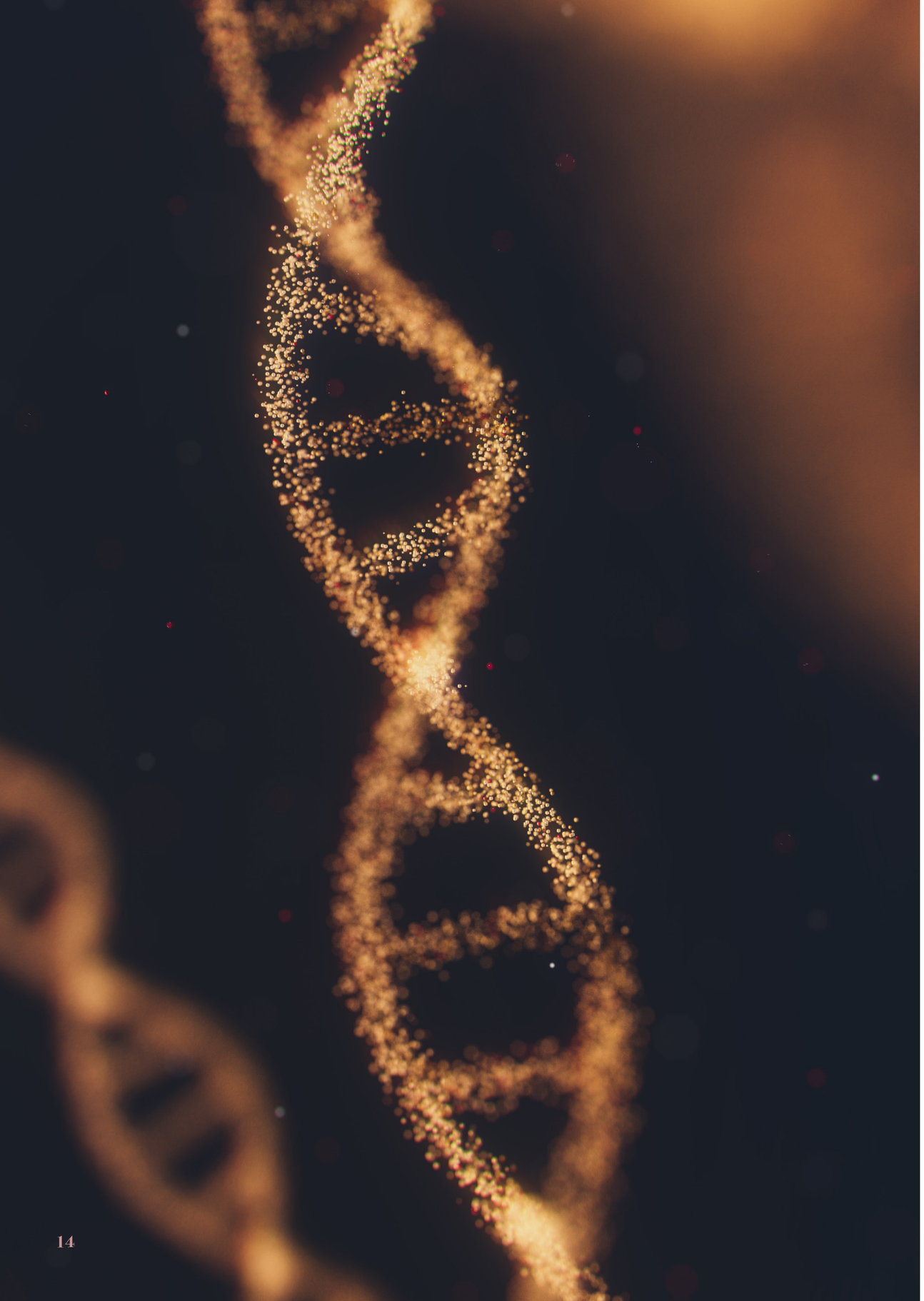

Design and Construction of a GAP-43 Reporter System for Potential Identification of Effective Therapeutics for Peripheral Nerve Regeneration

NINA CARLOS-DE CLERCQ, LISA BREEN, TOMMIE MCCARTHY

Abstract

BACKGROUND: Peripheral nerve injury (PNI) is a condition that can result in muscle paralysis and sensory disturbances. Electrical stimulation and/or the application of exogenous neurotrophic factors and cytokines are effective at enhancing nerve regeneration and is mediated via the expression of regeneration associated genes (RAGs) such as the growth associated protein GAP-43. Therapeutic upregulation of GAP-43 has potential use as a treatment for improving recovery from PNI. Few studies have investigated the potential of increasing GAP-43 for PNI therapeutic purposes and current methods for measuring GAP-43 expression are limited.

AIMS AND OBJECTIVES: The broader aim of this work was to construct a motor neuron-like cell model with a GAP-43 reporter system. Such a model would have potential use in screening for novel therapeutics that upregulate GAP-43 and in the optimisation of electrical stimulation treatment in combination with these therapies.

The key aim of the work was to design and construct a Cas9 expressing plasmid bearing a gRNA that targets GAP-43 cleavage and a donor plasmid bearing a reporter GFP or Neo cassette flanked with 5' and 3' GAP-43 homology arms (HAs) to facilitate the insertion of the reporter immediately 3' of the GAP-43 promoter via CRISPR/Cas9 homology directed repair (HDR). Such an insertion would enable quantitative measurement of endogenous expression of the GAP-43 gene.

METHODS AND RESULTS: To guide the Cas9 nuclease to the target location, GAP-43 gRNA oligomers were designed and cloned downstream of the U6 promoter in the Cas9 expression plasmid px330, which also expresses the Cas9 gene and the cloned gRNA when transfected into cells.

For CRISPR/Cas9 HDR, the 5' and 3' GAP-43 HAs were amplified from mouse genomic DNA and cloned into the donor plasmid using Gibson Assembly so that they flanked the reporter cassette.

RESULTS: This work successfully constructed the Cas9 gRNA expressing plasmid to target cleavage of the GAP-43 gene in mouse cell lines and has provided the complete design and construction foundation for generation of the reporter system for the endogenous GAP-43 gene in mice.

Introduction

Peripheral nerve injury is a condition that may result in muscle paralysis as well as sensory disturbances. In a study of 60,422 leg injuries, 1.8% of these patients suffered additional nerve trauma (1). Nerve injury has the highest level of socioeconomic expense due to the need for extensive rehabilitation (1). Peripheral nerves have the capability of repairing themselves after injury. However, natural nerve regeneration is slow (1-3mm/day) and has an increased risk of muscle atrophy leading to possible withdrawal of central nerve synapses (2).

In the case of neurotmesis, the axolemma (nerve cytoplasm) is exposed for a brief period to the extracellular environment. During this interval, ions are free to travel down their concentration gradients, leading to a calcium influx in both the proximal and distal stumps (5). In the proximal portion this calcium influx has a positive effect. It aids in the repair of the damaged axon membrane and sends a wave of depolarization toward the cell body (6). Once this wave reaches the cell body it causes an upregulation in the transcription of regeneration associated genes (RAGs) (2). In the distal stump, the calcium influx has the opposite effect. This portion is disconnected from the cell body, which is its source of energy and proteins. The lack of ATP means the sodium ATPases can no longer maintain equilibrium (7). A change in concentration gradient ensues and causes a reversal in the Na+/Ca++ exchanger channel, causing a further influx in calcium (5). This triggers Wallerian Degeneration in the nerve segment distal to the nerve injury (5). This type of degeneration clears the way for the regenerating neurites of the proximal stump to grow

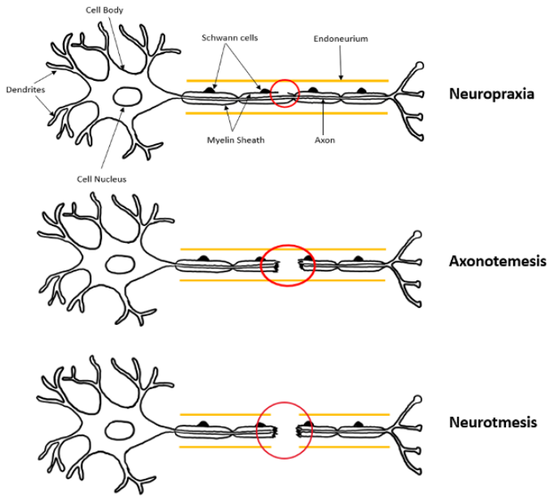

Figure 1. Illustration of Seddon's classification of nerve injury. In neuropraxia there is only mild demyelination, hence the nerve is capable of spontaneous recovery. Axonotmesis involves moderate demyelination and a severed axon. However, since the endoneurium is intact the nerve is capable of repair without treatment. Neurotmesis is a complete transection of the nerve where the myelin, axon, and endoneurium are all interrupted. An intact endoneurium is key to guiding the regenerating neurons, hence surgical repair is required to achieve adequate recovery. (4)

into the remaining endoneurium of the distal stump, eventually reinnervating the target organ (5).

The use of electrical stimulation at the time of surgical repair further aids the process of regeneration. In a study by Al-Majed et al. a transected nerve was given electrical stimulation succeeding surgical repair. The electrically stimulated nerve regenerated over a 25mm gap 5-7 weeks faster than the sham stimulated nerve (8). Electrical stimulation is thought to mimic the intracellular calcium wave that occurs in the proximal segment of the neuron after nerve injury (9).

Following nerve injury, several RAGs are upregulated (10). GAP-43 is an example of one of these genes (11). It is essential in nerve regeneration through the regulation of the nerve's cytoskeleton. The cytoskeleton functions as the internal scaffold of the nerve, maintaining its shape (12). A type of cytoskeleton involved in nerve growth are microfilaments, composed of actin monomers. GAP-43 plays a role in regulating these actin monomers. In the process of cytoskeleton assembly, GAP-43 promotes the clustering of phosphatidylinositol-4,5-bisphosphate (PIP2) (11). PIP2 is a phospholipid component of the cell membrane that is involved in cell signalling (12). Its accumulation leads to a recruitment of proteins that carry out actin polymerisation, increasing the nerve length (11).

Following nerve injury, GAP-43 expression is upregulated with a strong correlation to nerve regeneration (11, 13, 16, 17). Knockdown studies of GAP-43 in neurons resulted in reduced neurite outgrowth and impaired neuronal path finding (14, 16). Furthermore, in studies where GAP-43 was overexpressed nerve regeneration was enhanced (18). Hence, GAP-43 is a suitable indicator of successful peripheral nerve regeneration and can be used to measure the effectiveness of various therapies treating peripheral nerve regeneration.

One particular methodology that can be used to measure gene expression is a reporter system. Rojas et al. (2015) successfully engineered a reporter system downstream of a target gene using CRISPR/Cas9 (20). This method used a Cas9/gRNA nuclease complex to make a double stranded break in the endogenous genomic DNA, immediately 5' to the start codon (ATG) of the gene. The DNA is repaired via homology directed repair (HDR) using a donor vector with homology arms (HAs) flanking the reporter cassette as a template. There were two different reporter cassettes used throughout, one containing the 717bp Green Fluorescent Protein (GFP) and the other 792bp Neomycin resistance gene (Neo). The plasmids containing these cassettes were named pLucGFP and pLucNeo, respectively. GFP can be used to select for positive clones via Fluorescence-Activated Cell Sorting (FACS). Neo positive clones can be selected using G418 antibiotic selection. Both cassettes also contained the 558bp Gaussia luciferase (gLuc) reporter gene, the 587bp

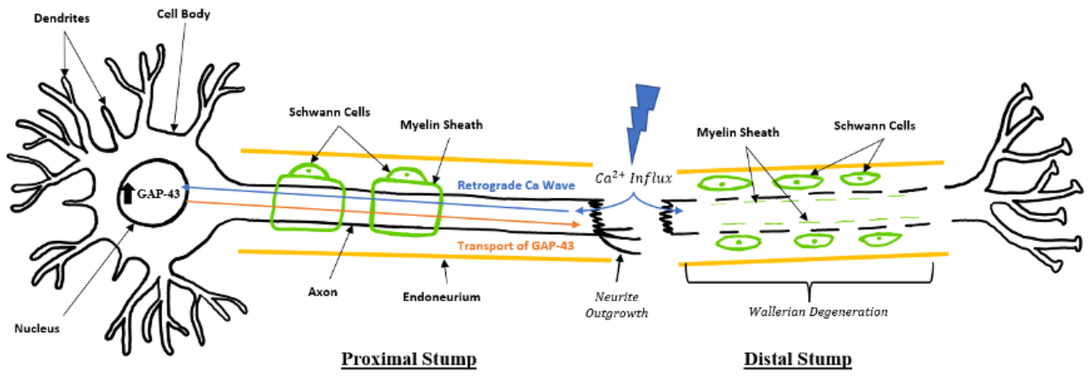

Figure 2. Schematic overview of the calcium influx effect following PNI. In the proximal stump the calcium influx aids in the repair of the membrane and sends a retrograde wave of depolarisation toward the cell body. This signals for the upregulation of GAP-43 which is sorted into vesicles for fast axonal transport to the injury site to aid in neurite outgrowth. In the distal stump an excessive influx of calcium triggers Wallerian Degeneration. This section of the nerve breaks down into its various components: Schwann cells, myelin sheath and axon. Macrophages are recruited to break down the myelin and axon debris, leaving the Schwann cells intact. The neurites continue to regenerate throughout the distal stump until the target organ is reached.

IRES element and the 54 bp 2a peptide. The gLuc is secreted from transfected cells, allowing changes in gene expression to be quantitatively measured by determining the luciferase activity in the cell culture media. IRES ensures separate translation of GFP/Neo and luciferase protein. The 2a peptide cleaves the GFP/Neo from the downstream GAP-43 protein, ensuring the proteins are produced independently.

The overall aim of this study is to serve as the first step in the development of a motor neuron-like cell model allowing efficient measurement of GAP-43 promoter activity. Such a model has potential use in screening for nerve repair therapeutics that upregulate GAP-43 and in the optimisation of electrical stimulation treatment in combination with these therapies. The key objectives are to design and construct both a Cas9 plasmid bearing gRNA that targets GAP-43 cleavage and a donor plasmid comprising of a reporter cassette to be inserted immediately 3' of the endogenous GAP-43 promoter using CRISPR Cas9 HDR. This work will focus on the mouse GAP-43 gene, as mouse motor neuron-like cell models are well developed in comparison with human cell model.

Materials and Methods

EXPERIMENTAL STRATEGY

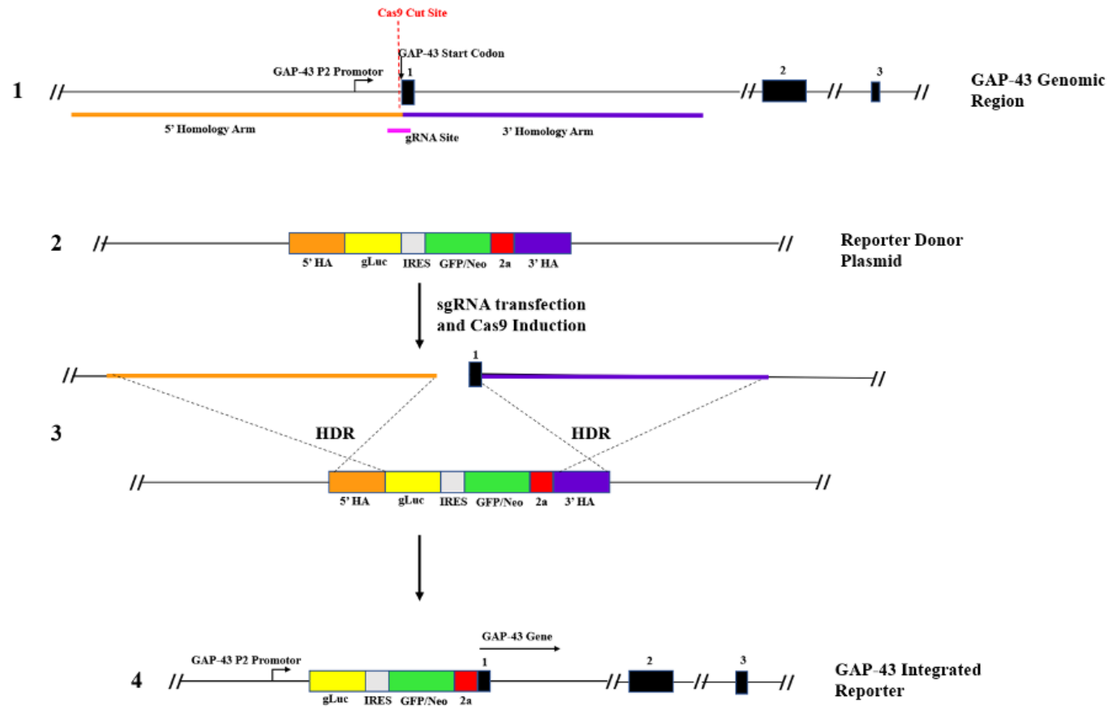

Figure 3. A schematic representation of the Rojas et. al. experimental strategy. (1) Genomic GAP-43 gene has three exons represented as black boxes. 5' and 3' GAP-43 HAs in the genome are represented by orange and purple lines, respectively. The targeted insertion site is directly 5' of the GAP-43 start codon. The gRNA site, in pink, overlaps the junction between the 5' and 3' GAP-43 HAs. The Cas9 cut site is located between the A and T of the ATG start codon. (2) The reporter donor vector including the 5' and 3' GAP-43 HAs, luciferase (Luc), Internal Ribosomal Entry Site (IRES), Green Fluorescent Protein (GFP)/Neomycin (Neo), and 2a peptide. (3) HDR of the DNA after cleavage via the gRNA/Cas9 nuclease complex, using the reporter donor plasmid as a template. (4) Reporter integrated into the GAP-43 locus.

Ligation of GAP-43 gRNA Sequence into the px330 Vector

ANNEALING OF GAP-43 gRNA OLIGOMERS

The GAP-43 gRNA target site was selected as close as possible to the reporter cassette insertion site (immediately 5' of the GAP-43 ATG start codon). The Benchling Design tool (https://www.benchling.com/) was used to design the gRNA site. The GAP-43 gRNA complimentary single strand oligomers were designed with BbsI overhangs to facilitate ligation to the BbsI digest px330 plasmid (Fig. 18.) The oligomers were ordered using IDT DNA oligo service (https://eu.idtdna.com/pages/products/custom-dna-rna/dna-oligos).

The gRNA complimentary oligomers (Table 1) were annealed by adding 1.5µL of each of the sense and antisense oligomers along with 5.0µL of 10X NEB T4 ligase buffer. The mixture was incubated in a thermocycler, at a setting of 95 degree celcius for 4 minutes. This was followed by a reduction in temperature of 0.1 degree celcius every second until room temperature was reached (26 degree celcius) and repeated for 8 cycles. Annealing reaction mixtures were analysed on a 1.5% agarose sodium borate gel, with a 1x sodium borate buffer at 200V for 10 minutes and a Solis BioDyne 100bp DNA ladder.

Table 1. Sequence of the GAP-43 gRNAs as oligomers. BbsI overhangs are highlighted in blue.

Name of RNA	RNA Sequence
gRNA-sense-Gap43	5'- CACCCCATGCTGTGCTGTATGAGA -3'
gRNA-antisense-Gap43	5'- AAACTCTCATACAGCACAGCATGG -3'

PREPARATION OF px330 VECTOR

1µg of the px330 plasmid was cut with 1.0µL BbsI-HF restriction enzyme in 5.0µL of 10X rCutSmartBuffer. The mixture was incubated at 37 degree celcius for 15 minutes. The cut px330 vector was analysed on a 0.8% agarose sodium borate gel, with a 1x sodium borate buffer at 100V for 40 minutes and a Solis BioDyne 1kbp ladder.

GOLDEN GATE ASSEMBLY OF GAP-43 gRNA DS OLIGO AND PX330 VECTOR

A ligation reaction of the annealed oligomers (1µL), the open px330 vector (3µL), 2µL T4 ligase and 2µL 10X ligase buffer was carried out. The mixture was incubated at 16 degree celcius for 3 hours.

TRANSFORMATION OF THE GAP-43 gRNA px330 RECOMBINANT PLASMID

The gRNA-px330 plasmid was then transformed into DH5alpha cells for amplification. Using 2µL ligation reaction with 25µL of DH5α cells. (Control was 2µL of cleaved px330 plasmid in place of the ligation reaction.) The cells were heat shocked at 42 degree celcius in a water bath for 90 seconds, followed by an incubation on ice for 2 minutes. Cells were recovered with 400µL of LB (Lysogeny Broth) and incubated for 1 hour at 37 degree celcius. The px330 backbone has ampicillin resistance allowing for the selection of successfully transformed cells. The transformants (150µL) were pipetted onto LB/Ampicillin plates and incubated at 37 degree celcius for 15 hours.

A miniprep kit (Thermofisher Scientific Genejet Miniprep Kit) was used to purify the gRNA-px330 plasmid from the cells and confirmed via sequencing, using the 'Eurofins Mix-2-Seq kit.'

Design and Assembly of the Reporter Donor Plasmid

DESIGN OF THE GAP-43 HOMOLOGY ARMS

To incorporate the GAP-43 HAs into the donor plasmid, Gibson Assembly was used. These HAs were designed to facilitate insertion of a reporter cassette immediately 5' of the GAP-43 start codon. The optimal HA length is 500-1000 base pairs (22), hence there was a long and short variation designed for each of the 5' and 3' HAs. The long and short 5' HAs designed were 969bp and 595bp respectively, and the 3' HAs 725bp and 530bp respectively.

Amplification of the 5' and 3' Homology Arms: the 5' and 3' GAP-43 homology arms forward and reverse primers were designed homologous to the insertion site (immediately 5' to the ATG start codon of GAP-43) (Table 2). The 5' GAP-43 homology arm forward and reverse primers had incorporated Gibson tails compatible with the BclI site of pLucGFP/pLucNeo plasmid and the 5' luciferase side of the reporter cassette, respectively. The 3' GAP-43 homology arm forward and reverse primers had incorporated Gibson tails compatible with the 3' T2A side of the reporter cassette and the BglII site of pLucGFP/pLucNeo plasmid, respectively. For PCR amplification 4 µL HOT FIREPol Blend Master Mix (HOT FIREPol DNA polymerase, proofreading enzyme, 5x Blend Master Mix Buffer, 1mM dNTPs of each, Bovine serum albumin) was used with 100ng mouse genomic DNA for the template. The PCR conditions were carried out as recommended by the HOT FIREPol protocol.

Table 2. Sequence of the homology arm primers for GAP-43. The lower case represents the Gibson assembly overlap. The upper case represents the annealing region primer

Name of Homology Arm Primers	Sequence
5' Homology Arm Forward	5′ gacggccagtgaattcacttTGCAGTGCGGAAAGTCAG 3′
5' Homology Arm Reverse	5′ aacagaactttgactcccatGGTGGTATCTTCCCCTGC 3′
3' Homology Arm Forward	5′ tggaggagaatcccggcccaATGCTGTGCTGTATGAGAAG 3′
3' Homology Arm Reverse	5′ cgactctagaggatccagtaCGAGCACGAAATCAGGTATC 3′

GIBSON ASSEMBLY OF THE REPORTER DONOR PLASMID

The donor plasmid was made by assembling the multi-use reporter donor vectors pLucGFP and pLucNeo. The vectors are comprised of a pUC19 backbone and either the Luc-IRES-GFP-T2a or Luc-IRES-Neo-T2a reporter cassette flanked by the GAP-43 5' and 3' homology arms. Gibson assembly primers were designed using the NEBuilder Gibson Assembly Fragment Calculator.

The pLucNeo and the pLucGFP plasmids were prepared using a BclI and BglII double restriction enzyme digest. Both the pLucNeo and the pLucGFP plasmids were first cut with 1µL Bgl-II restriction enzyme in 5µL 10X NE buffer. The pLucGFP was incubated with the enzyme for 2 hours, the pLucNeo for 2 hours 30 minutes. Restriction enzyme Bcl-I was then added to both mixtures and incubated for a further 15 minutes at 50 degree celcius. Restriction enzyme digest mixtures were analysed on a 0.8% agarose sodium borate gel, with a 1x sodium borate buffer at 100V for 40 minutes and a Solis BioDyne 1kbp DNA ladder.

These fragments were assembled by adding the relevant segments (5' and 3' homology arms of GFP/Neo) in equimolar amounts to a 15µL Gibson Assembly Master Mix (5x isothermal Master Mix, T5 exonuclease, Phusion DNA Pol, Taq DNA ligase) and incubated for 1 hour at 50 degree celcius.

TRANSFORMATION OF pLucGFP/NEO PLASMID

The Gibson Mixtures of the pLucNeo and pLucGFP were then transformed into DH5α cells. Each of the amounts in Fig 20 were added to separate Eppendorf's. The cells were heat shocked at 42 degree celcius in a water bath for 90 seconds, followed by an incubation on ice for 2 minutes. The cells were recovered using 400µL LB, and 150µL of the transformants were pipetted onto LB/Amp plates. The plates were incubated at 37 degree celcius for 15 hours.

COLONY PCR OF THE PLUCGFP/NEO TRANSFORMANTS

A successful Gibson assembly was indicated in select colonies by amplifying the 5' GAP-43 homology arm using each colony as a template in colony PCR (61.2 degree celcius annealing temperature). The PCR product for select plasmid colonies were analysed using a 1% agarose sodium borate gel, with a 1x sodium borate buffer at 100V for 40 minutes and a Solis BioDyne 100bp DNA ladder. Based on this result 3 colonies were swabbed and grown overnight in 5ml LB and 5µL Ampicilin for 17 hours. Followed by plasmid purification using 'Thermofisher Scientific Genejet Miniprep Kit'. A successful Gibson assembly was confirmed via sequencing using the 'Eurofins Mix-2-Seq kit.'

Results

1. Successful Construction of the GAP-43 gRNA px330 Recombinant Plasmid

AMPLIFICATION OF THE GAP-43 gRNA AND CLONING INTO THE px330 VECTOR PLASMID

GAP-43 gRNA sense and antisense oligomers were amplified and then annealed to generate double stranded gRNA. Results were assessed by agarose gel electrophoresis. Annealed oligomers migrated as a higher molecular weight (Fig. 4, lane 4).

The px330 plasmid was prepared for cloning of the double stranded GAP-43 gRNA by cutting with BbsI restriction enzyme (Fig. 5).

Ligation of the GAP-43 gRNA and px330 plasmid was performed successfully. The ligation mixture was transformed into DH5α E. coli cells. The GAP-43 gRNA px330 plasmid was prepared from 3 positive colonies. DNA sequencing of these plasmids confirmed the presence of the correct GAP-43-gRNA sequence in the px330 plasmid.

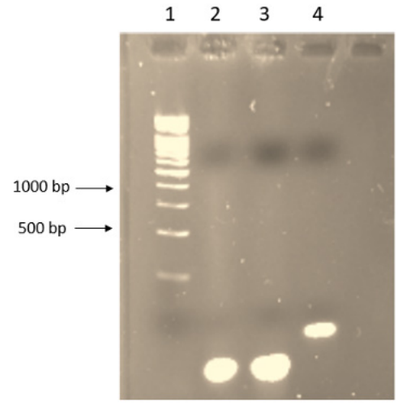

Figure 4. Analysis of GAP-43 gRNA annealed oligomers. Annealing was carried out by incubation of sense and antisense gRNA oligomers at 95 degrees celcius for 4 minutes followed by a reduction in temperature of 0.1 degrees celcius every second until 26 degrees celcius was reached. The mixture was analysed using agarose gel electrophoresis (1.5%) and stained with SafeView to allow visualisation. Lane 1, 100bp Solis BioDyne molecular weight ladder. Lane 2 and 3, 24bp sense gRNA oligomer and 24bp antisense gRNA oligomer. Lane 4, annealed gRNA oligomers.

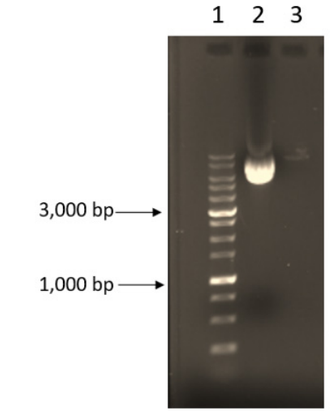

Figure 5. Analysis of px330 restriction enzyme digest. The 8484bp px330 plasmid was incubated with BbsI at 37 degrees celcius for 15 minutes. The mixture was analysed using agarose gel electrophoresis (0.8%) and stained with Safeview to allow for visualisation. Lane 1, 1kbp Solis BioDyne molecular weight ladder. Lane 2, px330 plasmid prior to BbsI restriction digest Lane 3, BbsI digested px330 plasmid.

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CACCCCATGCTGTGCTGTATGAGA          -Sequence of original gRNA
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CGAAACACCCCATGCTGTGCTGTATGAGAGTTTT -Sequence of recombinant px330gRNA plasmid
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Figure 6. Sequencing results of GAP-43 gRNA cloned into the px330 vector plasmid.

2. Construction of the Reporter Donor Plasmid

AMPLIFICATION OF THE GAP-43 HOMOLOGY ARMS AND CLONING INTO THE PLUCGFP/PLUCNEO DONOR PLASMID

All of the 5' and 3' GAP-43 HAs were successfully amplified (Fig 4.1). This was followed by a double restriction enzyme digest of the pLucGFP/pLucNeo reporter cassette plasmids with the BclI and BglII restriction enzymes that was verified by gel electrophoresis (Fig. 4.2). For Gibson Assembly of the digested pLucGFP/pLucNeo plasmids were incubated with 5' and 3' GAP-43 HAs in the presence of the Gibson Assembly master mix. While the long HAs were amplified successfully, their use in Gibson Assembly was unsuccessful, hence the short 5' and 3' GAP HAs were used throughout.

Following Gibson assembly, the master mix was transformed into DH5α E. coli cells and were plated on LB/ampicillin agar plates. Ten ampicillin resistant colonies were selected from the each of the pLucGFP and pLucNeo Gibson assembly product plates. Subsequently, colony PCRs amplifying the 5' GAP-43 HA were carried out to indicate positive clones. (Fig. 4.3). DNA sequencing of 3 selected colonies from each reaction confirmed the presence of the final the 5' and 3' GAP-43 HA in the pLucGFP/pLucNeo GAP-43 reporter donor plasmid. These plasmids were named p5G43pLucGFP3G43 and p5G43pLucNeo3G43 (Appendix B, Fig. 14, 15).

SEQUENCE ALIGNMENT OF THE 5' HA FOR P5G43PLUCGFP3G43 AND P5G43PLUCNEO3G43

DNA sequence alignment of the cloned HAs with the reference sequence was carried out using CLUSTAL Omega. Sequence changes were observed in the 5' HA differed from the mouse genomic reference sequence. These are highlighted in yellow (Fig. 5). In the 5' GAP-43 HA of the p5G43pLucNeo3G43 plasmid there were four point mutations observed along with several mutations around position 360-420 where the pattern of bases was repeated a number of times (Fig. 5). However, in the p5G43pLucGFP3G43 plasmid there were only 2 point mutations noted.

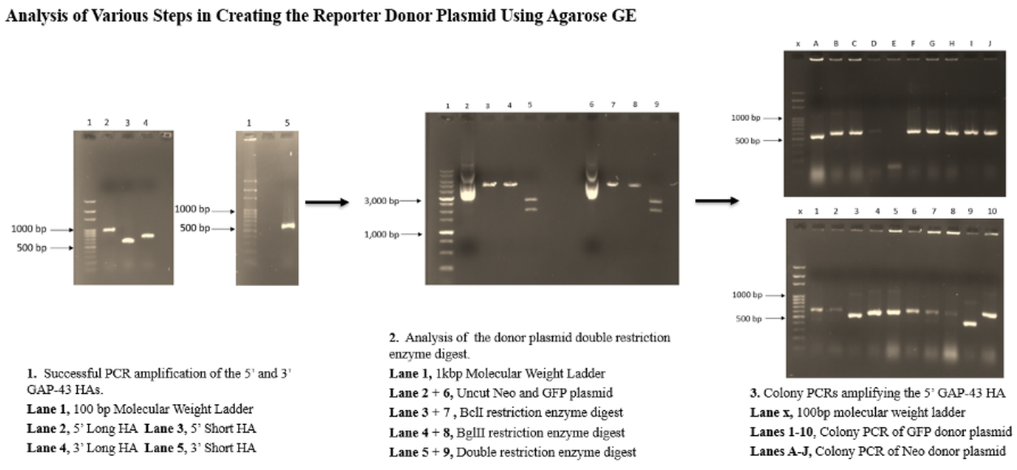


Figure 7. Analysis of the steps in the construction of the Donor Plasmid using Agarose Gel Electrophoresis

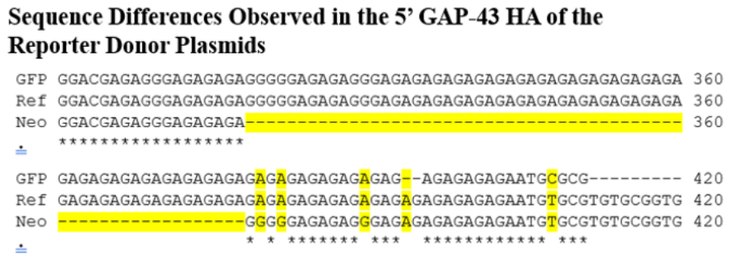


Figure 8. Sequence differences observed in the 5' GAP-43 HA of the GAP-43 GFP and Neo reporter donor plasmids.

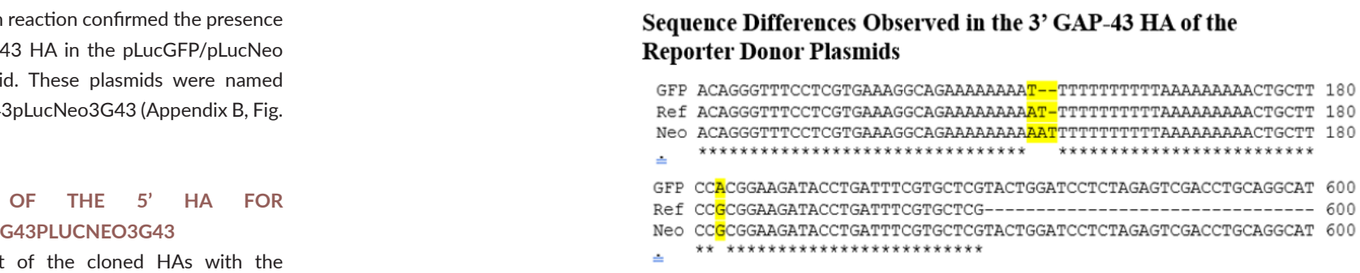


Figure 9. Sequence differences observed in the 3' GAP-43 HA of the GAP-43 GFP and Neo reporter donor plasmid

Discussion and Conclusions

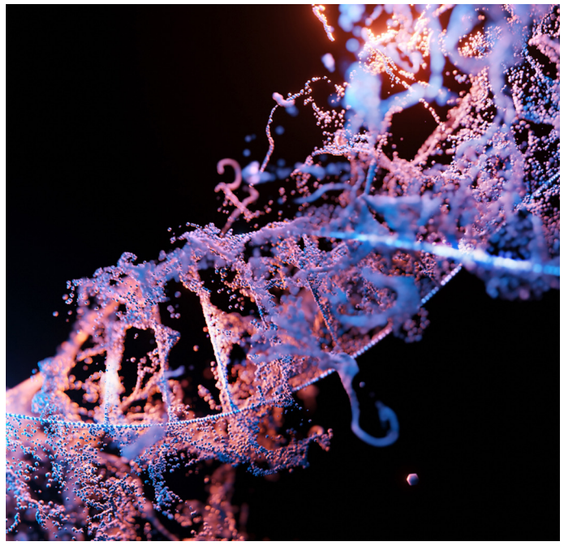
Recombinant plasmids containing the key elements required for Cas9 mediated insertion of a reporter into the GAP-43 gene were successfully generated in this project. The assembly of the Cas9 gRNA component for the cleavage of GAP-43 into px330 plasmid was achieved and the sequence of gRNA in the px330 plasmid was also validated. The GAP-43-gRNA px330 plasmid contained no sequence errors. Hence, it is ready for use in transfection into the mouse cell line to generate the GAP-43 target cleavage necessary for the insertion of the reporter cassette by CRISPR/Cas9 HDR.

Following the construction of the reporter donor plasmid, sequencing errors were observed in the cloned 5' and 3' GAP-43 HAs. They are believed to have occurred in the amplification process and reflect DNA replication errors. These errors should be readily amended by repeating this work using a high-fidelity PCR amplification protocol. Most mutations observed occurred in regions with runs of As and repeated sequences, where PCR amplification errors are common (23). The polymerase used for the work reported here was a standard type of Taq polymerase with an approximate error rate of 1 base in 4000 amplified. The amplification of the GAP-43 HAs should be repeated using a DNA polymerase such as Q5 polymerase which has a higher fidelity with a 10-20 fold reduced error rate. Regardless of the sequence errors, this project has successfully provided the design of DNA primers for the amplification of the 5' and 3' GAP-43 HAs and has validated that these primers successfully amplify the HAs selected. It has also demonstrated that these HAs can be successfully incorporated into the pLucGFP and pLucNeo plasmids.

The design of this work is aimed at measuring GAP-43 expression from its P2 promotor, as it is 10 times more active than P1 (14). To further investigate the promoter activity of GAP-43, a replica of the project design with relevant modifications has the potential for insertion of the reporter under the control of the GAP-43 P1 promoter.

Potential cell lines for the next stage of this project were reviewed. A promising primary cell line is the NSC-34 cell line. The cell line was recommended by S. Geuna et al. who reviewed various in vitro models for peripheral nerve regeneration (24). The NSC-34 cells are consistent with motor neuron properties such as generating action potentials and the production of acetyl choline (24). In addition, the NSC-34 cells express GAP-43 making the cell line an ideal candidate for the insertion of the reporter system (25) for use in screening novel therapeutics that upregulate GAP-43.

The approach outline has high prospects for identifying novel therapeutics for upregulating GAP-43 for enhancing peripheral nerve regeneration. Electrical stimulation (ES) is an accessible therapy that can be used following surgical repair of a damaged nerve (2, 8, 11). It encourages earlier and more sustained upregulation of GAP-43 (2, 8). This reporter system model could be used for the optimisation of ES treatment on PNI. The dose dependent application of exogenous factors such as BDNF and IL-6 to injured nerves has exhibited improvements in nerve regeneration in previous studies (27, 28, 29). The reporter system of GAP-43 may be used to investigate the optimal dosage of these factors to treat PNI. Finally, there are limited studies investigating the combination of electrical stimulation and the application of exogenous factors such as BDNF and IL-6 to treat PNIs. The GAP-43 reporter system has the potential to be used to discover multimodal treatments producing optimal GAP-43 upregulation for nerve regeneration.



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Appendix 1: Sequence Alignment Results of the GAP-43 Homology Arms

SEQUENCE ALIGNMENT OF THE 5' HA FOR p5G43pLucGFP3G43 AND p5G43pLucNeo3G43

DNA sequence alignment of the cloned HAs with the reference sequence was carried out using CLUSTAL Omega. Sequence changes were observed in the 5' HA differed from the mouse genomic reference sequence. These are highlighted in yellow (Fig. 14, 15). In the 5' GAP-43 HA four point mutations were observed along with several mutations around position 360-420 where the pattern of bases was repeated a number of times.

```
GFP GGGGTTTCAGTCACGACGTTGTAAACGACGGCCAGTGAATTCACTTTGCAGTGCGGAAAG 60
Ref ------------------------------------------------TGCAGTGCGGAAAG 60
Neo --------GGTCACGACGTTGTAAACGACGGCCAGTGAATTCACTTTGCAGTGCGGAAAG 60
.                                                   **************

GFP TCAGTGGGTAATTGGGTCCAGATTGGAGGTGTTTAAATATTCATGAGGCTGGCAGGGGAC 120
Ref TCAGTGGGTAATTGGGTCCAGATTGGAGGTGTTTAAATATTCATGAGGCTGGCAGGGGAC 120
Neo TCAGTGGGTAATTGGGTCCAGATTGGAGGTGTTTAAATATTCATGAGGCTGGCAGGGGAC 120
.   ************************************************************

GFP TGGGAGGGGGTGACTGTCTAGAAATGGGGGTAGGGGCTACGGGAAGTGATTAGTCACTGG 180
Ref TGGGAGGGGGTGACTGTCTAGAAATGGGGGTAGGGGCTACGGGAAGTGATTAGTCACTGG 180
Neo TGGGAGGGGGTGACTGTCTAGAAATGGGGGTAGGGGCTACGGGAAGTGATTAGTCACTGG 180
.   ************************************************************

GFP AAGCTAGCAAACAATTCTGAGAAAGGGACCCAGGGAGAAGGAAGAAAAAGATTGGGTGGG 240
Ref AAGCTAGCAAACAATTCTGAGAAAGGGACCCAGGGAGAAGGAAGAAAAAGATTGGGTGGG 240
Neo AAGCTAGCAAACAATTCTGAGAAAGGGACCCAGGGAGAAGGAAGAAAAAGATTGGGTGGG 240
.   ************************************************************

GFP GAGTGGAGGAAAGAGGAGAAGGAAGGAAGGAAAAGGAGAGAGGAAGGAAAGAGGAGGAAG 300
Ref GAGTGGAGGAAAGAGGAGAAGGAAGGAAGGAAAAGGAGAGAGGAAGGAAAGAGGAGGAAG 300
Neo GAGTGGAGGAAAGAGGAGAAGGAAGGAAGGAAAAGGAGAGAGGAAGGAAAGAGGAGGAAG 300
.   ************************************************************

GFP GGACGAGAGGGAGAGAGAGGGGGAGAGAGGGAGAGAGAGAGAGAGAGAGAGAGAGAGA 360
Ref GGACGAGAGGGAGAGAGAGGGGGAGAGAGGGAGAGAGAGAGAGAGAGAGAGAGAGAGA 360
Neo GGACGAGAGGGAGAGAGA----------------------------------------- 360
.   ******************

GFP GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG--AGAGAGAGAATGCGCG--------- 420
Ref GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAATGTGCGTGTGCGGTG 420
Neo ------------------GGGGGAGAGAGGGAGAGAGAGAGAGAATGTGCGTGTGCGGTG 420
.                     * * ******* ***  ************ ***

GFP ------------------------------------------------------------ 480
Ref AGCAATAGCTGTGGACCTTACAGTTGCTGCTAACTGCCCTGGTGTGTGTGAGGGAGAGAG 480
Neo AGCAATAGCTGTGGACCTTACAGTTGCTGCTAACTGCCCTGGTGTGTGTGAGGGAGAGAG 480

GFP ------------------------------------------------------------ 540
Ref AGAGAGAGAGGGAGAGGGAGGGAGAGGGAGGGAGGGAGGGAGAGCGCGCTAGCGCGAGAG 540
Neo AG---------------------------------------------------------- 540
```

Figure 10. Sequencing results of the amplified 5' forward GAP-43 HA from the reporter donor plasmids (5' reverse was the same). The 5' HA sequence obtained from the p5G43LucGFP3G43 (GFP) and p5G43LucNeo3G43 (Neo) were aligned with a mouse DNA reference sequence (ref) using CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)

SEQUENCE ALIGNMENT OF THE 3' HA FOR p5G43pLucGFP3G43 AND p5G43pLucNeo3G43

In the 3' GAP-43 HA two mutations were observed. At position 180 there was a deletion of a T and an addition of an AT evident in GFP and Neo respectively. Furthermore, a single base mutation was apparent in GFP at position 600, where an A replaces a G.

```
GFP TCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCAATGCTGTGCTGTATGAGAAGAAC 60
Ref ------------------------------------ATGCTGTGCTGTATGAGAAGAAC 60
Neo TCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCAATGCTGTGCTGTATGAGAAGAAC 60
.                                       **********************

GFP CAAACAGGTAGAGCTAAAGATTTCTTTTTTACTTCTTTGCTGTTGTGAAATTATCAGAGT 120
Ref CAAACAGGTAGAGCTAAAGATTTCTTTTTTACTTCTTTGCTGTTGTGAAATTATCAGAGT 120
Neo CAAACAGGTAGAGCTAAAGATTTCTTTTTTACTTCTTTGCTGTTGTGAAATTATCAGAGT 120
.   ************************************************************

GFP ACAGGGTTTCCTCGTGAAAGGCAGAAAAAAAAT--TTTTTTTTTTAAAAAAAAACTGCTT 180
Ref ACAGGGTTTCCTCGTGAAAGGCAGAAAAAAAAT-TTTTTTTTTTAAAAAAAAACTGCTT 180
Neo ACAGGGTTTCCTCGTGAAAGGCAGAAAAAAAAATTTTTTTTTTTAAAAAAAAACTGCTT 180
.   ******************************   ************************

GFP CTGCCCTGGCATGATGCTCTGGCTTCCTTAGCATACGGTAACTGATGCTTGCATCCCGGC 240
Ref CTGCCCTGGCATGATGCTCTGGCTTCCTTAGCATACGGTAACTGATGCTTGCATCCCGGC 240
Neo CTGCCCTGGCATGATGCTCTGGCTTCCTTAGCATACGGTAACTGATGCTTGCATCCCGGC 240
.   ************************************************************

GFP GTTATTCTTTTCTGCCTTTCATGATCTGGTTTTTGGAATGCTGCTACTAATTAGGGTAAG 300
Ref GTTATTCTTTTCTGCCTTTCATGATCTGGTTTTTGGAATGCTGCTACTAATTAGGGTAAG 300
Neo GTTATTCTTTTCTGCCTTTCATGATCTGGTTTTTGGAATGCTGCTACTAATTAGGGTAAG 300
.   ************************************************************

GFP GGGAGAGAAATATGCCGGCTTGGCTAGAAATATGATTCCCCTCGCCTATTAGTAAGTGCT 360
Ref GGGAGAGAAATATGCCGGCTTGGCTAGAAATATGATTCCCCTCGCCTATTAGTAAGTGCT 360
Neo GGGAGAGAAATATGCCGGCTTGGCTAGAAATATGATTCCCCTCGCCTATTAGTAAGTGCT 360
.   ************************************************************

GFP CAGCCGCTAGGTCTCTGGTTTTGAGGGTGTGGATGCAGAAAGGGGTGTGGGGACGATGTG 420
Ref CAGCCGCTAGGTCTCTGGTTTTGAGGGTGTGGATGCAGAAAGGGGTGTGGGGACGATGTG 420
Neo CAGCCGCTAGGTCTCTGGTTTTGAGGGTGTGGATGCAGAAAGGGGTGTGGGGACGATGTG 420
.   ************************************************************

GFP GGCTCTATCTACGAGATCAAAAAAGCTAATCTTGATATTATTTGTGGAAAATTAGGTCTG 480
Ref GGCTCTATCTACGAGATCAAAAAAGCTAATCTTGATATTATTTGTGGAAAATTAGGTCTG 480
Neo GGCTCTATCTACGAGATCAAAAAAGCTAATCTTGATATTATTTGTGGAAAATTAGGTCTG 480
.   ************************************************************

GFP GGGGAATTATAGTCACATTTCAACATTGCCTGTTCCGTGATTCAAATTTTCTCACATGTG 540
Ref GGGGAATTATAGTCACATTTCAACATTGCCTGTTCCGTGATTCAAATTTTCTCACATGTG 540
Neo GGGGAATTATAGTCACATTTCAACATTGCCTGTTCCGTGATTCAAATTTTCTCACATGTG 540
.   ************************************************************

GFP CCACGGAAGATACCTGATTTCGTGCTCGTACTGGATCCTCTAGAGTCGACCTGCAGGCAT 600
Ref CCGCGGAAGATACCTGATTTCGTGCTCG------------------------------- 600
Neo CCGCGGAAGATACCTGATTTCGTGCTCGTACTGGATCCTCTAGAGTCGACCTGCAGGCAT 600
.   ** ************************
```

Figure 11. Sequencing results of the amplified 3' forward GAP-43 HA from the reporter donor plasmids (3' reverse was the same). The 3' HA sequence obtained from the p5G43LucGFP3G43 (GFP) and p5G43LucNeo3G43 (Neo) were aligned with a mouse DNA reference sequence (Ref) using CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)

Appendix 2: Sequence Maps of the Reporter Donor Plasmids

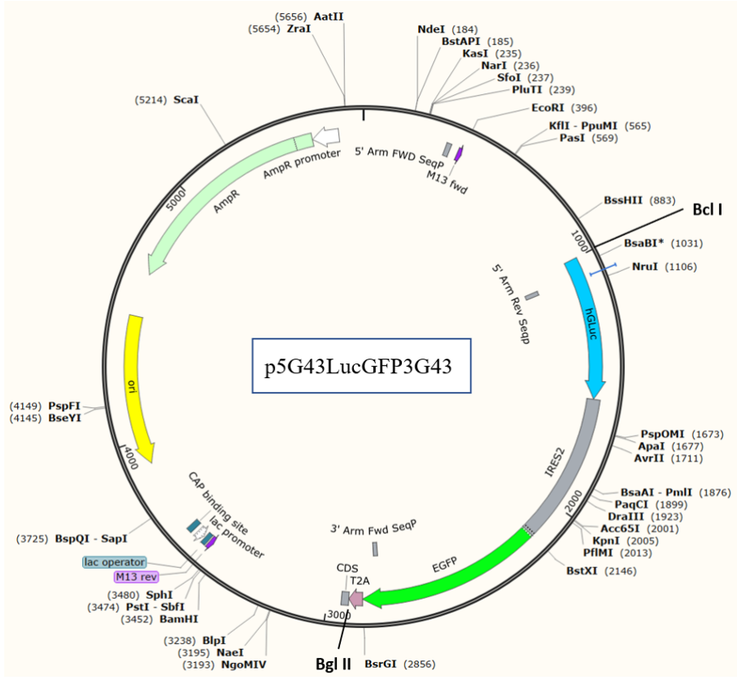

Figure 12. Map of the p5G43LucGFP3G43 reporter donor plasmid with the BclI and the BglII sites indicated.

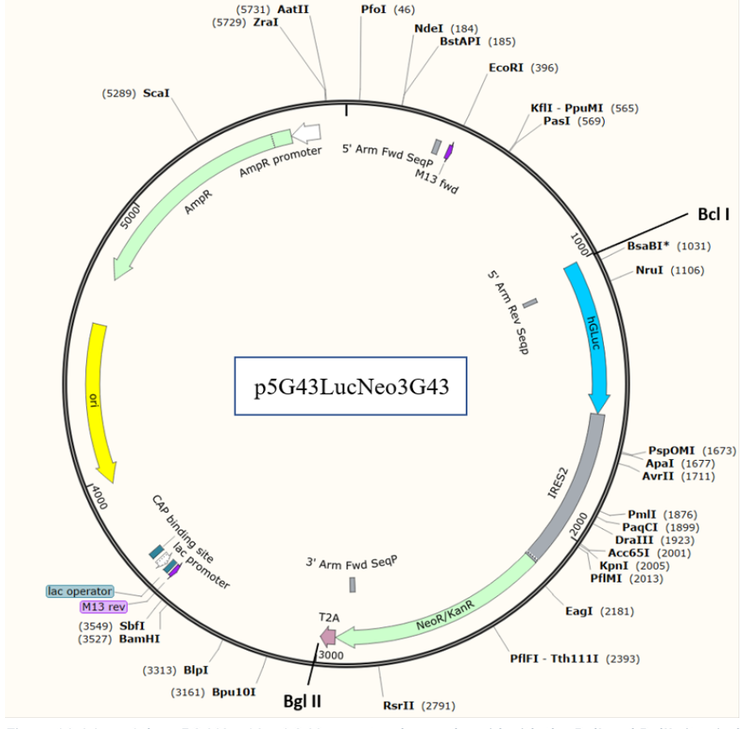

Figure 13. Map of the p5G43LucNeo3G43 reporter donor plasmid with the BclI and BglII sites indicated.