Evaluation of rationally designed synthetic promoters for expression in neurons and glial cells

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AVIADOBIO vMiX™

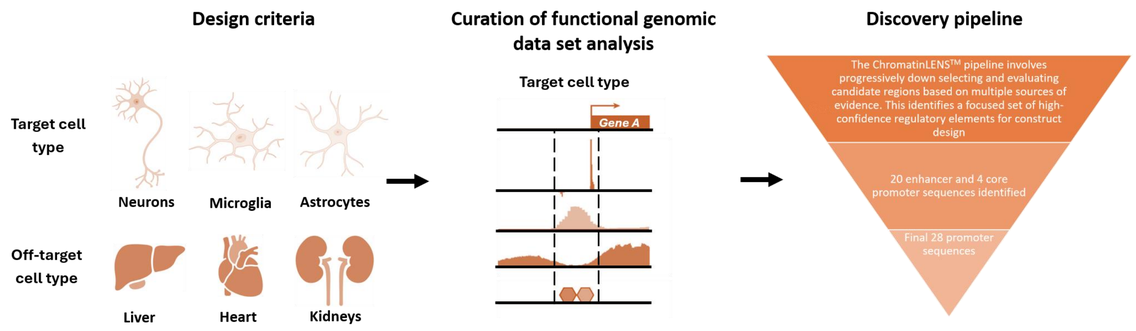
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Introduction

- AviadoBio's (AVB) proprietary vMiX™ platform makes use of miRNA sequences, vectorised into a rAAV capsid, to downregulate pathogenic genes. In the context of neurodegenerative conditions, such as Alzheimer's disease and amyotrophic lateral sclerosis, vMiX™ offers a potential means of reducing expression of associated disease-related genes, with the aim of slowing neurodegeneration. Whilst pathology is largely synonymous with neuronal dysfunction and cell loss, glial cells are increasingly understood to play a key role in disease pathogenesis[1].
- AVB collaborated with Chromatin Bioscience (CHB) to design novel promoters engineered to display transcriptional activity within neurons and glia. In addition to a stringent on-target profile, these promoters were designed to exhibit minimal activity in peripheral tissues to facilitate use in systemically delivered therapeutics, targeting the CNS.
- This study evaluated the activity of 28 candidate promoters, alongside standard reference plasmids containing the hSYN1 and CB7 promoter sequences.

Promoter design

Figure 1: CHB promoter design using ChromatinLENS™



- CHB sought to engineer compact, low-GC promoters to drive high expression in neurons, astrocytes, and microglia while minimising expression in off-target tissues such as the heart and liver (**Figure 1**).
- Using the ChromatinLENS™ platform, CHB compiled and curated a myriad of multi-omics datasets, identifying four candidate promoter sequences and 20 enhancer elements specific for neurons and glial cells.
- Utilising this information, CHB rationally constructed a panel of 28 promoter sequences.

In vitro assessment – eGFP expression and knock down in cell lines

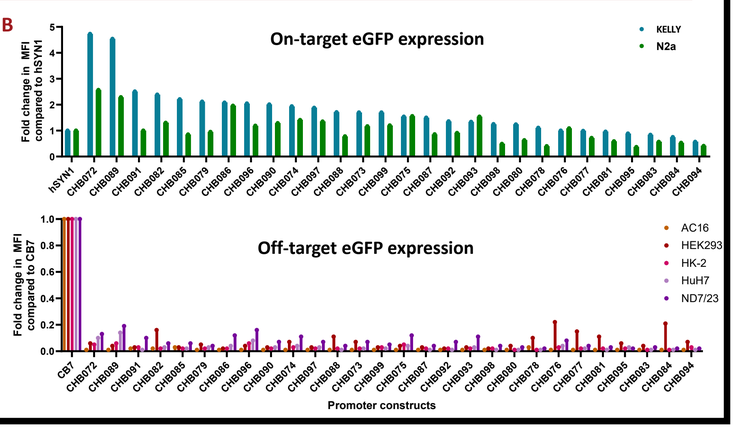
- The 28 candidate promoter sequences were positioned immediately upstream of an *eGFP* reporter gene. Each expression cassette was flanked by AAV2 ITRs. Control plasmids harbouring canonical CB7 and hSYN1 promoter sequences were constructed in parallel. Additionally, a transfection control plasmid containing the *smURFP* reporter gene driven by the CB7 promoter was prepared.
- The 28 promoters, alongside the control plasmids, were transfected into a panel of "on-target" and "off-target" cell lines to assess eGFP expression (**Figure 2A**).
- Cells were assessed by means of flow cytometry. The proportion of eGFP-positive cells relative to the whole singlet cell population and mean fluorescent intensity (MFI), was used to differentiate between the transcriptional activity of the test promoters. In the "on-target" cells, the test promoters were compared to hSYN1, and to CB7 in the "off-target" cells (**Figure 2B**).
- 10 of the CHB promoters led to enhanced eGFP production by at least two-fold in KELLY cells. Promoters CHB072 and CHB089 were found to elicit over 4.5-fold greater eGFP expression in KELLY cells, relative to hSYN1, and over 2-fold in the N2A cells.
- All promoters displayed transcriptional activity at least 4-fold lower than the CB7 promoter in the "off-target" cells.

Figure 2: eGFP expression from CHB promoters in on-target and off-target cell lines

A

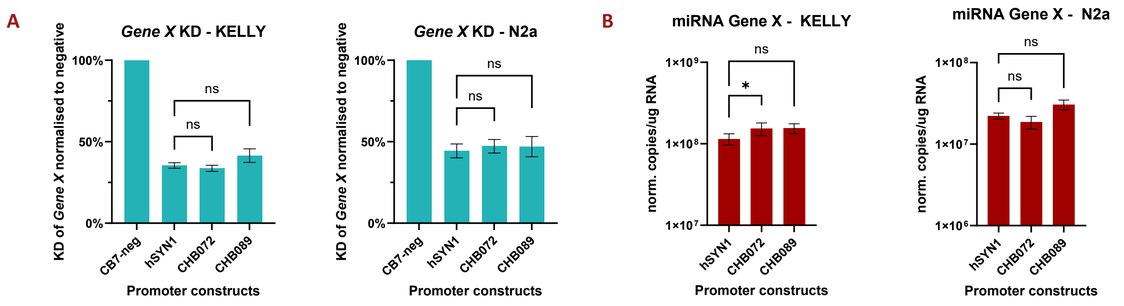
Cell line	Description
KELLY	Human neuroblastoma cells
N2a	Mouse neuroblastoma cells
AC16	Human ventricular cardiac cells
HEK293	Human embryonic kidney epithelial cells
HK-2	Human kidney proximal tubular cells
HuH7	Human hepatocarcinoma cells
ND7/23	Fusion of rat dorsal root ganglion neurons and a mouse neuroblastoma cell line

B



- The top performing promoters from the initial screen, CHB072 and CHB089, were cloned into AVB's vMiX™ miRNA cassette targeting *Gene X*. These constructs were transfected into KELLY and N2a cells, alongside analogous plasmids harbouring the hSYN1 and CB7 promoters. *Gene X* knockdown and miRNA expression, were quantified by RT-dPCR and miRT-dPCR respectively.
- Both CHB072 and CHB089 promoters were found to bring about *Gene X* knockdown, comparable to that of the hSYN1 promoter, in both KELLY and N2a cells (**Figure 3A**). The test promoters also elicited miRNA expression at a level comparable to the hSYN1 promoter, in both cell lines (**Figure 3B**).

Figure 3: Evaluation of CHB072 and CHB089 in vMiX™ in on-target cell lines

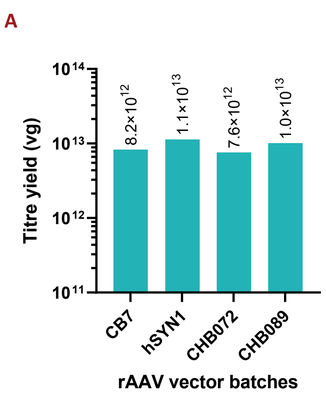


Manufacturability assessment

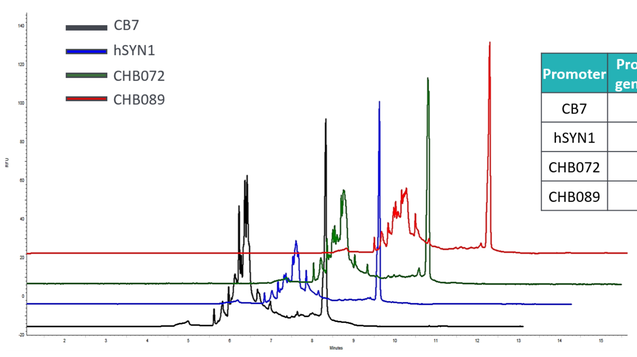
- Following the initial *in vitro* screen, the eGFP expression cassettes harbouring CHB072, CHB089, CB7 or hSYN1 promoters, were packaged into rAAV capsids. Vectors were manufactured in HEK293 suspension cells and purified by means of ÄKTA affinity chromatography and anion-exchange chromatography.
- Viral genome titre was quantified for each purified batch via dPCR. Total vector yield was found to be comparable between all four AAV preparations (**Figure 4A**).
- These vector batches were characterised using CE-GI LIF (**Figure 4B**).
- The rAAV genome housing the CB7 promoter demonstrated a greater propensity for truncation, compared to genomes housing the other promoters.
- The proportion of DNA-containing rAAV particles housing the full-length genome, was found to be largely comparable between those harbouring the hSYN1 and CHB072 sequences. CE-GI analysis of the rAAV vector containing the CHB089 promoter demonstrated a higher percentage of full-length genome compared to vector material housing the hSYN1 promoter.

Figure 4: Vector titration and truncation analysis

A



B

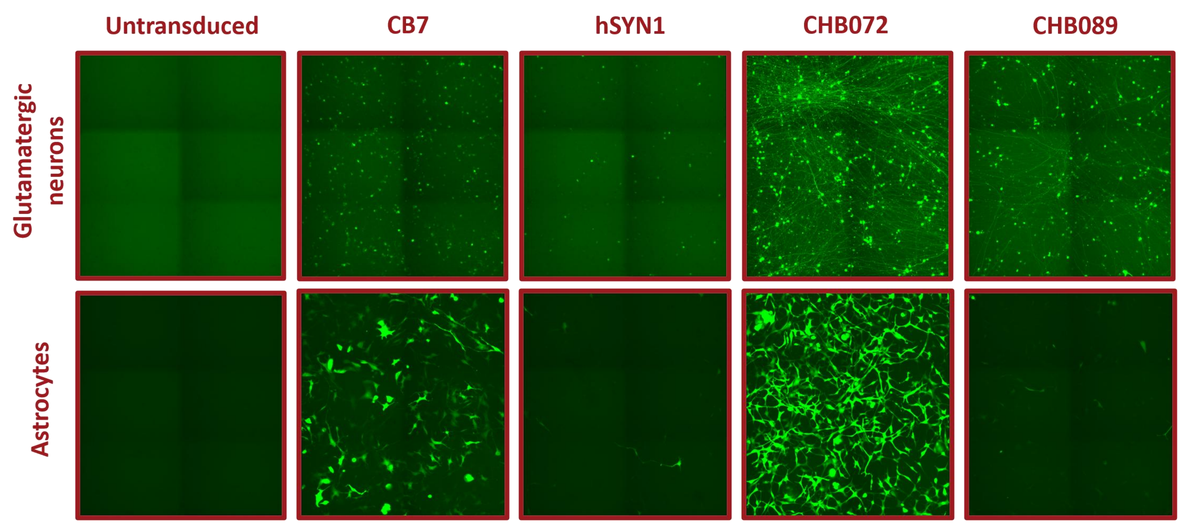


Promoter	Proportion of full-length viral genome normalised to hSYN1
CB7	0.65
hSYN1	1.00
CHB072	1.07
CHB089	1.44

In vitro assessment – eGFP expression in human iPSC-derived cells

- iPSC-derived glutamatergic neurons and astrocytes were cultured to maturity according to the manufacturer's protocols. Upon maturation, cells were transduced at matched MOIs with rAAV harbouring the CB7, hSYN1, CHB072, or CHB089 promoters – neurons and astrocytes were transduced at MOIs of 1.0E+7 and 1.0E+6, respectively.
- Six days post-transduction, eGFP expression was assessed by fluorescence microscopy. Both CHB072 and CHB089 promoters were found to bring about a greater level of eGFP expression, relative to the hSYN1 and CB7 control promoter, in glutamatergic neurons (**Figure 5**).
- Astrocytes transduced with vectors housing the CHB072 promoter displayed a greater eGFP expression than those cells transduced with material housing the CB7, hSYN1 and CHB089 promoters.
- Negligible eGFP expression was observed in astrocytes transduced with vectors harbouring either CHB089 and hSYN1 promoters.

Figure 5: eGFP expression from CHB072 and CHB089 in iPSC-derived cells



Conclusions

- As part of a collaboration with CHB, we have identified two promoters, CHB072 and CHB089, which appear to exhibit transcriptional activity superior to that of the neuronal-specific hSYN1 promoter, in immortalised neuronal cell lines.
- These promoters also displayed minimal transcriptional activity in a panel of "off-target" cell lines.
- Manufacturability of vector harbouring the CHB089 or CHB072 sequences was found to be akin to that of material housing the hSYN1 promoter.
- Furthermore, the CHB072 promoter has also been demonstrated to display activity greater than that of CB7 in both iPSC-derived glutamatergic neurons and astrocytes.
- Both candidate promoters are to be further evaluated *in vivo.*
- Based on this initial data, CHB are to perform an iterative design strategy to generate a further panel of promoters, comprising of novel arrangements of the highest-performing enhancer elements and core promoter motifs, as identified in this work.
- This work represents a means of targeting both neurons and astrocytes, cell types involved in the pathogenesis of several neurodegenerative conditions, thus potentially augmenting the efficacy of our therapeutics.

REFERENCES: [1]Franklin, H., Clarke, B. E., & Patani, R. (2021). Astrocytes and microglia in neurodegenerative diseases: Lessons from human in vitro models. *Progress in Neurobiology*, *200*, 101973. Figure 1A was created using BioRender.com. The statistical test performed in Figure 3 is Dunn's multiple comparison test. ns = not significant; * $p < 0.05$. Bars represent the mean for Figure 2 (n = 3), and the mean ±SEM for Figure 3 (n = 3).

ABBREVIATIONS: **rAAV**: recombinant adeno-associated virus; **CB7**: Cytomegalovirus-enhanced chicken Beta-actin promoter version 7; **CE-GI LIF**: capillary electrophoresis-laser-induced fluorescence genome integrity; **dPCR**: digital polymerase chain reaction; **eGFP**: enhanced green fluorescent protein; **GC**: guanine cytosine; **HEK293**: human embryonic kidney cells; **hSYN1**: human synapsin I; **iPSC**: induced pluripotent stem cells; **ITR**: inverted terminal repeat; **kb:** kilobase; **KD**: knock down; **miRNA**: microRNA; **MFI**: mean fluorescent intensity; **MOI:** multiplicity of infection; **RT-PCR**: reverse transcription-polymerase chain reaction; **smURFP**: small ultra red fluorescence protein; **vg**: vector genome.

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