Methylation of rAAV vector DNA is not a mechanism for differential transgene RNA expression from rAAV gene therapy in mouse and NHP liver

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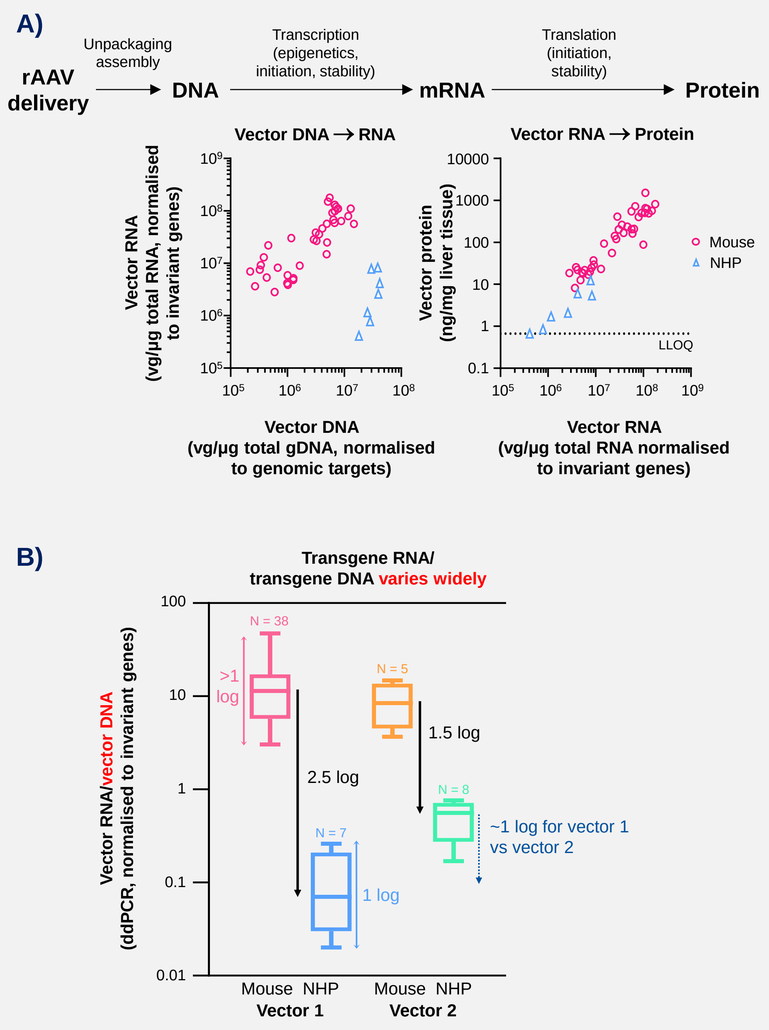
Introduction

- Understanding the mechanism(s) of variability in transgene expression within and between species is important for improved prediction and translation of recombinant adeno-associated virus (rAAV) gene therapy to the clinic
- In mouse and non-human primate (NHP) models, variability in vector transcription (DNA to RNA) is greater than in vector translation (RNA to protein), and transcriptional efficiency (expressed as RNA/DNA) is lower in NHP than in mice (**Figure 1**)
  - Remarkably, transcriptional efficiency varies by a factor of more than 500 between these species

Figure 1. Variability in protein expression from rAAV vectors across species is related to transcription. A) Vector transcriptional and translational efficiency in mice and NHP and B) with multiple vectors



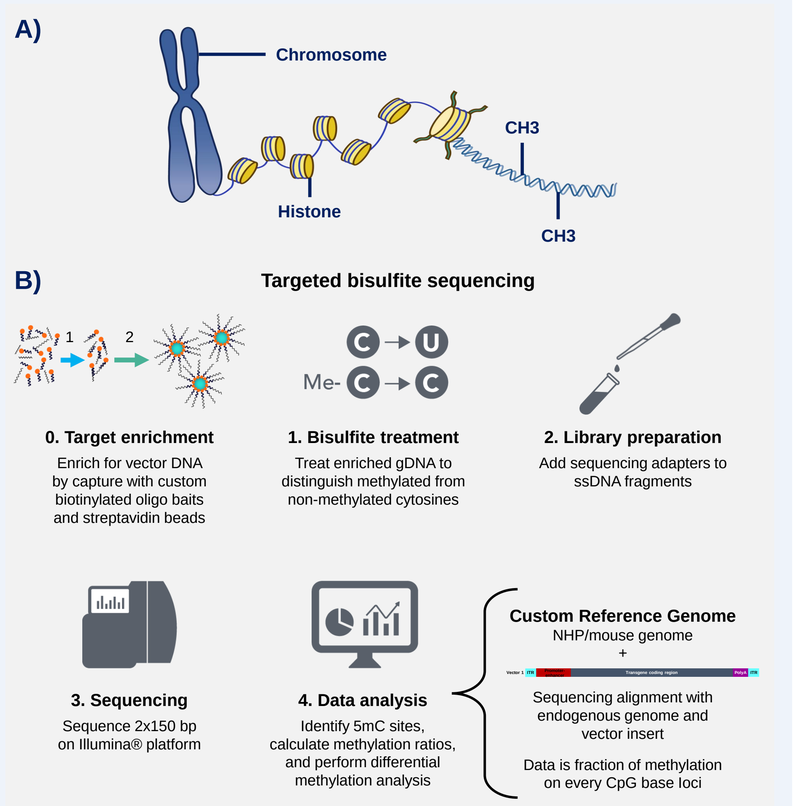
Mice and NHP were dosed with vector 1 or vector 2 as described in the methods section below. Data in panel A are from vector 1. ddPCR, droplet digital polymerase chain reaction; gDNA, genomic DNA; LLOQ, lower limit of quantitation; NHP, non-human primate; rAAV, recombinant adeno-associated virus; vg, vector genomes.

- Chromatin structure is regulated by many epigenetic modifications that influence transcriptional activity,[1] and rAAV vector DNA forms a chromosome-like structure with a nucleosome-associated pattern[2]
- We investigated vector DNA methylation as a possible mechanism to explain the intra- and inter-species variability in transcriptional efficiency
  - We hypothesized that CpG methylation in vector regulatory regions would inversely correlate with RNA expression

Methods

- Mice were dosed with $2x10^{13}$, $6x10^{13}$, or $2x10^{14}$ vg/kg, and NHP with $1x10^{14}$ or $4x10^{14}$ vg/kg of an rAAV vector construct containing a liver-selective promoter. Liver tissue was collected at 4, 12, 24, and 52 weeks after dosing
- Bisulfite treatment was performed on samples enriched for vector DNA and sequencing data was aligned to endogenous genome and the vector (**Figure 2**). Results are expressed as the fraction of methylated CpG on the vector as a whole and at the individual base resolution
- Vector DNA and RNA levels were quantified using droplet-digital PCR

Figure 2. Bisulfite genomic sequencing can compare differential patterns in methylated DNA. A) Methylated DNA structure. B) Bisulfite sequencing method



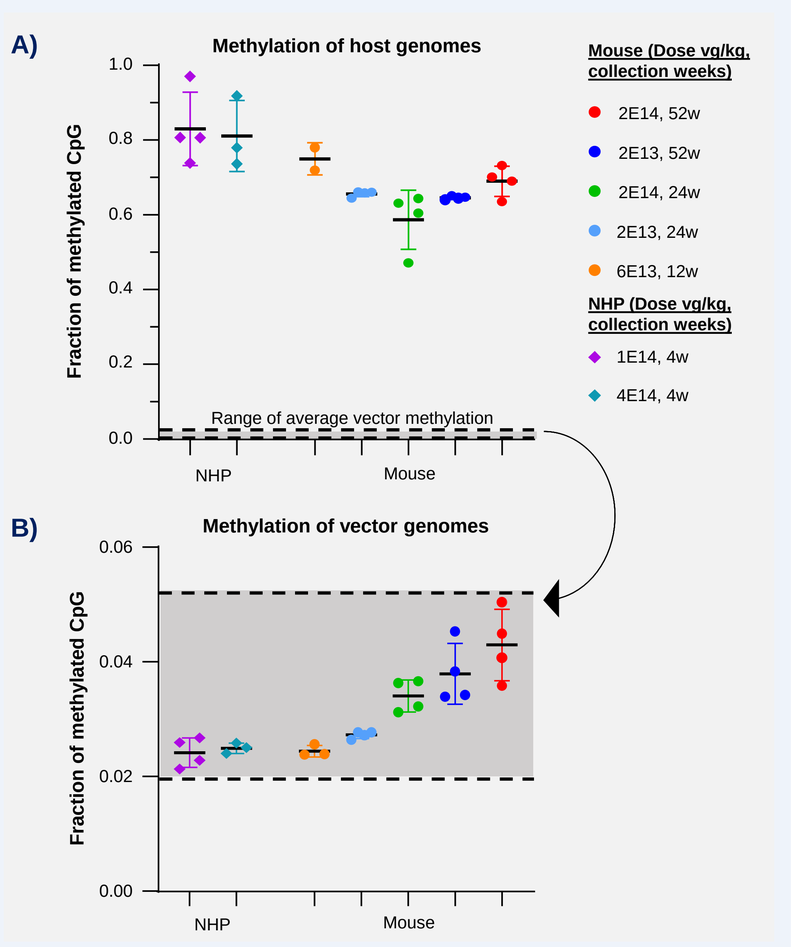
5mc, 5-Methylcytosine; bp, base pair; CH3, methyl group; CpG, cytosines followed by guanine residues; Me, methylated; NHP, non-human primate; gDNA, genomic DNA; ssDNA, single-stranded DNA.

Results

Intra- and inter-species variability in vector DNA methylation patterns

- Average DNA methylation is 10- to 20-fold lower on the vector compared to the genomic DNA (**Figure 3**)
- While vector methylation increases over time in mice, levels still remain >10-fold lower than the average level of genomic methylation

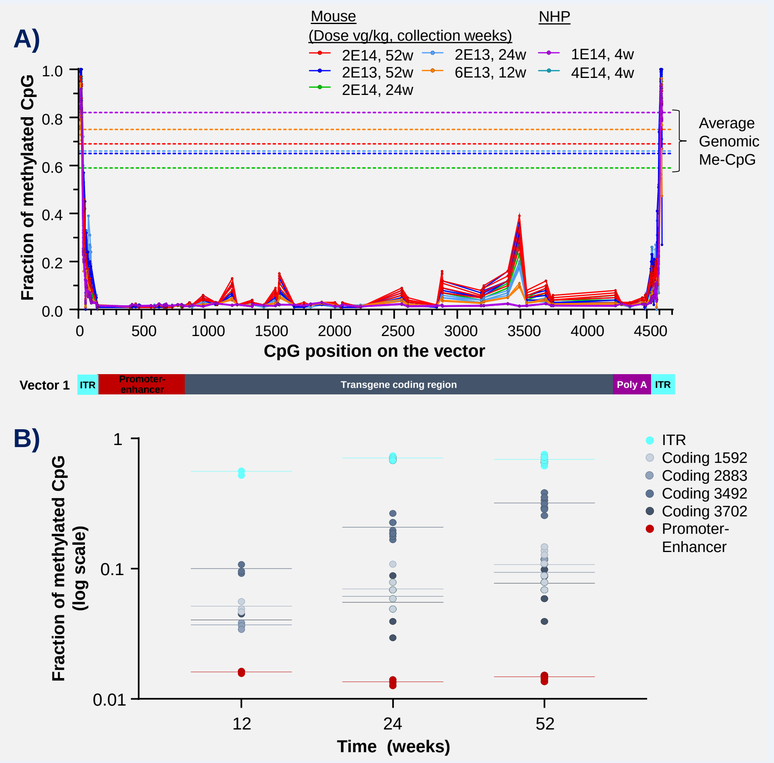
Figure 3. Average methylation in vector DNA is >10-fold lower than in genomic DNA. Fraction of methylated A) Genomic and B) Vector CpG residues



Data are from vector 1 (as shown in Figure 1), and averages methylation of CpG sites across the vector, excluding ITR regions. Dashed line marks the range of the average fraction of methylated CpG on the vector.
CpG, cytosines followed by guanine residues; ITR, inverted terminal repeat; NHP, non-human primate; w, week.

- Methylation at CpG residues on the vector is especially low in the enhancer-promoter region, and is similar between mice and NHP (**Figure 4**)
  - Methylation at CpG residues was highest at the inverted terminal repeats (ITR)
- While the fraction of methylated sites on the vector in the promoter-enhancer and ITR regions remained constant, methylation at sites within the coding region increased with time

Figure 4. Methylation frequency across the vector. A) Methylation pattern across the entire vector. B) Methylation pattern across key regions of the vector

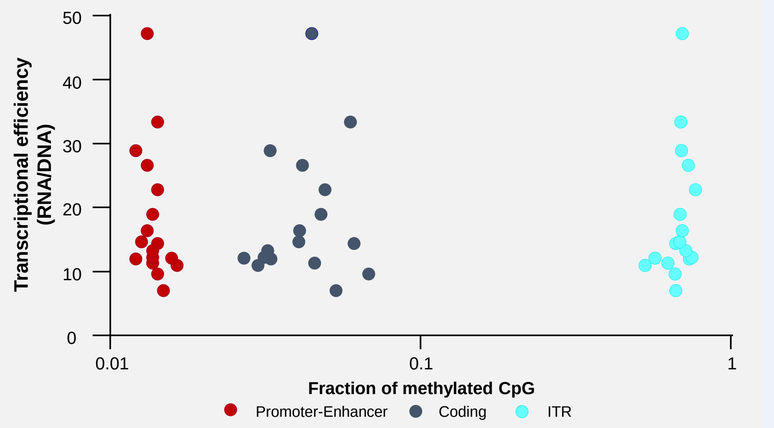


In panel B, data are from mice and include the same time points and doses shown in Figures 3 and 4A. Coding number is a CpG base position on vector.
CpG, cytosines followed by guanine residues; NHP, non-human primate; ITR, inverted terminal repeat; Me, methylated; PolyA, polyadenylation sequence; w, week.

Vector DNA methylation does not correlate with vector expression

- The average vector DNA methylation did not correlate with vector expression, and transcriptional efficiency was independent of vector DNA methylation (**Figure 5**)
- The observed increase in methylation patterns with time at coding regions did not correlate with vector expression

Figure 5. Transcriptional efficiency of vector is independent of vector DNA methylation in any region



Data are from mice using vector 1 (as shown in Figure 1) and include the same time points and doses shown in Figures 3 and 4A. Fraction of methylated CpG is an average value from each region of vector.
CpG, cytosines followed by guanine residues; ITR, inverted terminal repeat.

Conclusions

- Most vector CpG sites are methylated at very low levels compared with host chromosomal CpG methylation
- Vector elements are treated differently by the host DNA methylation machinery: ITR proximal CpGs are highly methylated, protein coding sequences are lightly methylated, and promoter-enhancer sequences are minimally methylated
- Coding sequence CpG methylation increases coordinately at several sites over time, while promoter-enhancer stays at basal levels, and does not correlate with expression levels
- Our analysis of the rAAV vector methylation patterns and RNA expression do not support a role for methylation in regulating transcriptional efficiency within or between species

References

1.Klemm et al. (2019) *Nat Rev Genet* 20(4):207–20.
2.Penaud-Budaloo et al. (2008) *J Virol* 82(16):7875–85.

Disclosures

All authors are current employees and stockholders of BioMarin Pharmaceutical Inc.

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