Investigating mechanisms of variability of AAV5-hFVIII-SQ expression in vitro

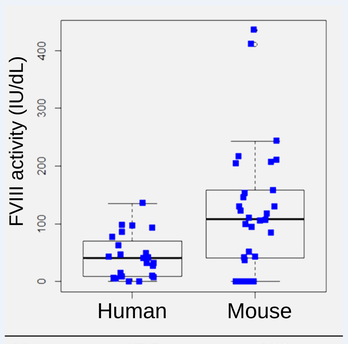
Bridget Yates, William Keenan, Lisa Razon, Stephen Scheeler, Su Liu, Stuart Bunting, Sylvia Fong

BioMarin Pharmaceutical Inc., Novato, CA, USA

Introduction

- Valoctocogene roxaparvovec (AAV5-hFVIII-SQ) is an approved gene therapy for the treatment of severe haemophilia A (HA)
- Similar to other adeno-associated virus (AAV)-mediated gene transfers, inter-subject variability has been observed in AAV5-hFVIII-SQ gene expression across species and trials; however, the mechanistic drivers of variability remain unknown
- Inter-subject variability observed in human factor VIII (hFVIII) expression in humans and haemophilic mice treated with AAV5-hFVIII-SQ is comparable (**Figure 1**)
- Analysis of human liver biopsy samples from participants enrolled in the phase 1/2 valoctocogene roxaparvovec clinical trial revealed inter-individual variability in transgene expression following successful transduction may result from differences in expression of regulatory molecules involved in transcription and protein folding/secretion[1]
- We hypothesise that variation in host factors involved in AAV serotype 5 (AAV5) transduction, vector genome trafficking, metabolism, hFVIII RNA expression, and hFVIII protein expression/secretion contribute to inter-subject variability
- We systematically investigated and identified 15 host factors whose expression levels correlated with AAV5-hFVIII-SQ expression in mice
- Here, we performed additional studies using a HepG2 in vitro hepatocyte model to identify drivers of AAV5 gene therapy variability

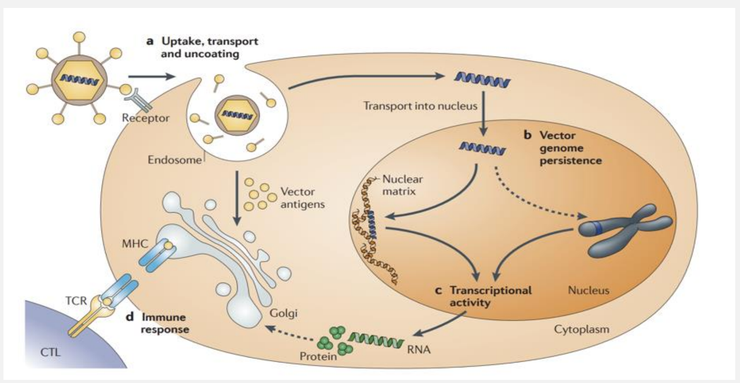
Figure 1. Inter-subject variability in man and mice



	Human	Mouse
Mean	47.5	122
SD	37.8	109
% CV	79.5	89.8

CV, coefficient of variation; FVIII, factor VIII; SD, standard deviation.

Variability is likely driven by multiple mechanisms



Kay M. *Nat Rev Genet.* 2011;12:316–28.
CTL, cytotoxic T cell; MHC, major histocompatibility complex; TCR, T cell receptor.

Potential mechanisms	
Transduction	• **Anti-AAV or other plasma factors** • **Non-hepatocyte capture of AAV** • **Vector uptake** • **Uncoating** • **Repair/episome assembly** • **Retention of DNA**
Transcript expression	• **Variations in host factors mediating AAV transcription** • **Epigenetic regulation of transgene** • **mRNA stability and clearance**
Cell capacity to produce exogenous proteins	• **Variations in host factors necessary to fold and secrete protein (mounting unfolded protein response)**
Other	• **Inflammation and cell-mediated immunity** • **Intrinsic and extrinsic patient factors**

AAV, adeno-associated virus; mRNA, messenger RNA.

Methods

In vivo methods

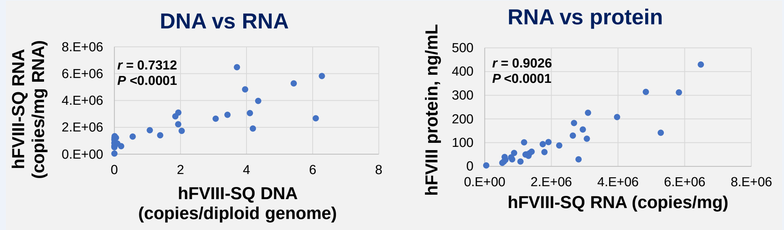
- Male C57BL6 mice were administered a single vector dose (1x10^{13} to 2x10^{14} vg/kg) of valoctocogene roxaparvovec by tail vein injection. Plasma FVIII protein levels and liver FVIII DNA, RNA, protein, and various markers were analysed

In vitro methods

- HepG2 cells were transduced with lentivirus (multiplicity of infection [MOI] 5) containing a short-hairpin RNA (shRNA) targeting genes of interest correlated with transgene levels (**Table 1**) to knockdown expression. After 48 hours, cells were transduced with AAV5-hFVIII-SQ (MOI 1E6). DNA and RNA were harvested on day 4 after AAV transduction
- AAV5-hFVIII-SQ vector genomes and RNA transcripts were quantified using droplet-digital polymerase chain reaction
- Knockdown of target gene mRNA was analysed both on the day of AAV5-hFVIII-SQ transduction and at final harvest

Results

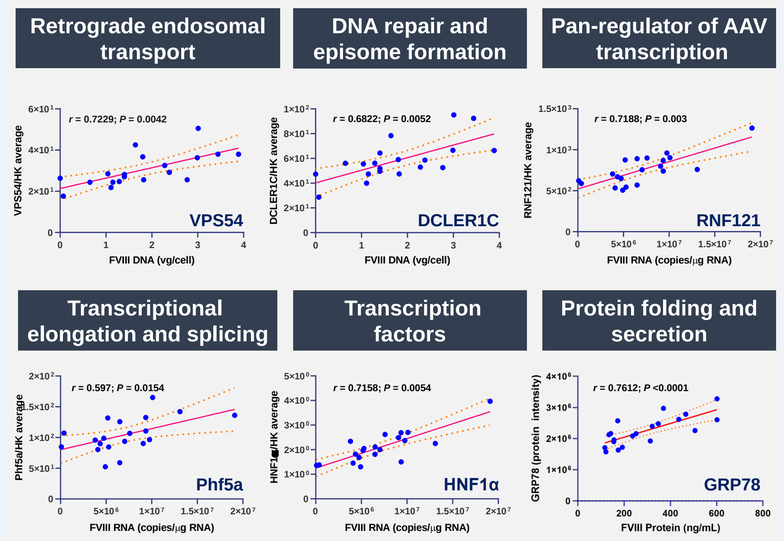
Figure 2. Hepatocyte transduction can affect inter-individual variability in mice



hFVIII, human factor VIII; hFVIII-SQ, hFVIII SQ variant.

- At doses of 1–3x10^{13} vg/kg (producing therapeutic FVIII levels), significant correlations were noted between levels of vector DNA and FVIII RNA, and between levels of liver FVIII RNA and circulating FVIII protein, suggesting multiple processes from vector uptake, intracellular trafficking, genome processing to retention, and transcription may play a role in inter-subject variability (**Figure 2**)

Figure 3. Molecules involved in retrograde endosomal transport, episome formation, transcription, and folding and secretion may contribute to AAV5-hFVIII-SQ variability



Data shown are Pearson correlation coefficient (r). *P*-values were not adjusted for multiple comparisons.
AAV, adeno-associated virus; FVIII, factor VIII.

Correlation analysis identified genes whose expression levels significantly correlated with either AAV5-hFVIII vector genome, transcript levels, or circulating FVIII protein

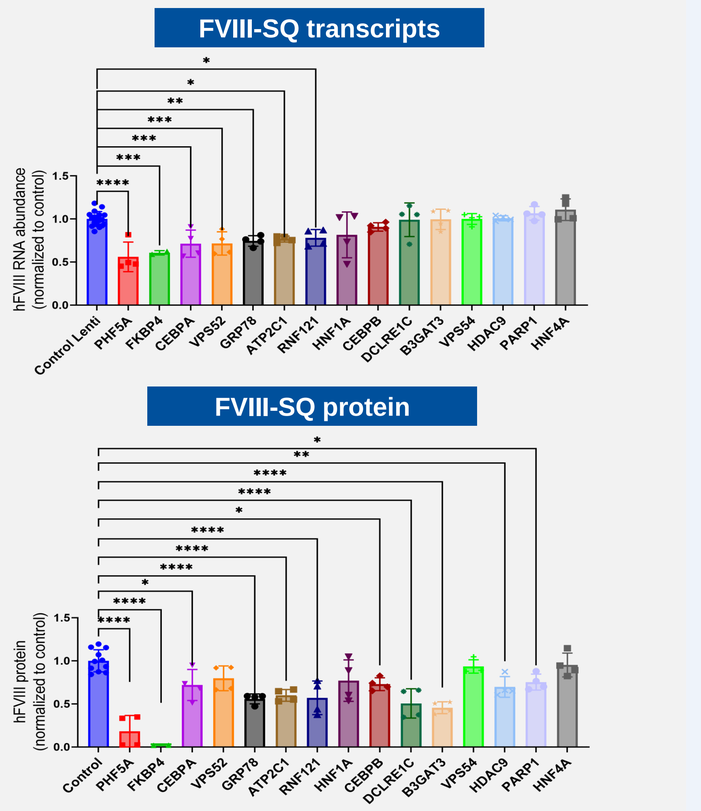
- **AAV trafficking:** Expression levels of genes involved in retrograde transport from early and late endosomes to the trans-Golgi network, VPS52 and VPS54 (two subunits of the Golgi-associated retrograde protein complex), correlate with FVIII-SQ vector DNA
- **Episome formation:** A panel of 86 DNA repair genes were screened and mRNA levels of Artemis, for example, significantly correlated with levels of FVIII-SQ vector DNA in mouse liver
- **Transcriptional machinery:** Genes, including a pan-regulator of AAV transcription (RNF121), splicing factor (Phf5A), as well as several liver specific transcription factors (HNF1α), were identified to correlate with FVIII-SQ transcript levels
- **Secretion:** A strong correlation between levels of Grp78, a chaperone protein responsible for folding and secreting proteins, and plasma FVIII protein levels was observed[2]

Table 1. Host factors whose expression levels correlated with AAV5-hFVIII-SQ transgene expression in mice

Candidate	Description	r value	*P*-value
HSPA5 (GRP78)	Chaperone and master regulator of the UPR	0.76	<0.0001
B3GAT3	Glycosylation of surface proteins	0.59	0.016
ATP2C1	Involved in ionic homeostasis of Golgi	0.67	0.005
HDAC9	Deacetylates histones resulting in epigenetic repression	**	
DCLRE1C	Involved in DNA recombination and double-strand break repair	0.68	0.005
VPS52	Involved in retrograde transport from early and late endosomes to the trans-Golgi network	0.68	0.001
VPS54		0.72	0.0042
PHF5A	Pre-mRNA splice factor. Involved in transcriptional elongation	0.60	0.0154
FKBP4	Inhibits viral second-strand DNA synthesis. Also involved in protein folding and trafficking	0.67	0.0047
CEBPA	Potential transcriptional factors that bind the AAV5-hFVIII-SQ promoter	0.72	0.00046
CEBPB		0.60	0.015
RNF121	mRNA synthesis from AAV capsid-associated genomes is markedly decreased in RNF121 KO cells	0.72	0.003
HNF1A	Transcription factors that bind to AAV5-hFVIII-SQ promoter	0.72	0.0006
HNF4A		0.43	0.059
PARP1	Poly-ADP-ribosyltransferase that mediates poly-ADP-ribosylation of proteins and plays a key role in DNA repair	0.64	0.03

**HDAC9 identified in human biopsy analysis.[1]
P-values were not adjusted for multiple comparisons.
ADP, adenosine diphosphate; AAV, adeno-associated virus; mRNA, messenger RNA; UPR, unfolded protein response.

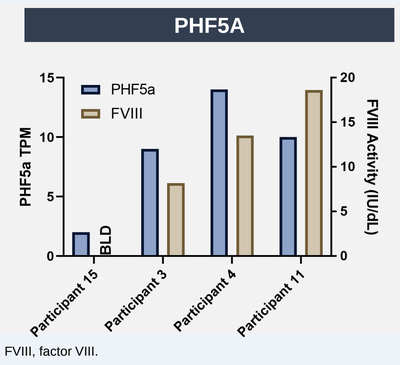
Figure 4. shRNA knockdown of FKBP4 and PHF5A in HepG2 cells significantly decreases FVIII-SQ RNA expression and secreted FVIII protein levels



****P* <0.0001, ****P* <0.001; ***P* <0.01; **P* <0.05 using a one-way ANOVA with a Dunnett's multiple comparisons test.
Data are shown as mean ± standard deviation.
ANOVA, analysis of variance; FVIII, factor VIII; hFVIII, human FVIII; hFVIII-SQ, hFVIII SQ variant; shRNA, short-hairpin RNA.

- Six out of 15 genes screened significantly reduced both AAV5-hFVIII-SQ transcript and hFVIII protein levels in HepG2 cells when knocked down using shRNA (**Figure 4**)
- FKBP4 has previously been shown to inhibit second strand synthesis of viral genomes and be involved in basic protein folding and secretion.[3] shRNA knockdown of FKBP4 reduced FVIII-SQ RNA levels by ~40% and FVIII protein levels by >98%
- PHF5A is involved in transcriptional elongation and splicing. shRNA knockdown of PHF5A in HepG2 cells significantly reduced both FVIII-SQ transcript and FVIII protein levels

Figure 5. Expression profile of PHF5A in human liver biopsies



BLD, below limit of detection; FVIII, factor VIII.

- Variability of PHF5A expression levels in human liver could be a host factor that contributes to inter-subject variability. RNA sequencing analysis of human liver biopsy samples from participants in the phase 1/2 valoctocogene roxaparvovec clinical trial identified low levels of PHF5A in a low responder[1] (**Figure 5**)

Conclusions

- Overall, we demonstrated that multiple host-mediated factors could contribute to variability of AAV5-hFVIII-SQ expression in mice
- Knockdown of genes involved in second strand synthesis, transcription, and protein folding and secretion resulted in significant reductions in transgene RNA and protein
- Further work to elucidate mechanistic drivers and mechanisms underlying inter-subject variability may help identify predictive biomarkers of response and approaches to optimise outcomes of AAV gene therapy

References

1. Fong S, et al. *Nat Med.* 2022;28:789–97. 2. Fong S, et al. *Mol Ther Med Clin Dev*. 2020;18:620–30. 3. Dai Q, et al. *Nucleic Acid Res*. 2008;36:7136–45.

Disclosures

All authors are employees or former employees and stockholders of BioMarin Pharmaceutical Inc.

Acknowledgements

BioMarin Pharmaceutical Inc. provided funding for the study and data analysis. Editorial support was provided by Kathleen Pieper, PhD, of AlphaBioCom, LLC, and funded by BioMarin Pharmaceutical Inc.