

Nonviral Plasmid-Delivered Follistatin Can Last at Least One Year in Humans

Running title: Follistatin gene therapy in healthy adults

Walter Patterson¹, Ryan Rossner¹, Raven Garuda¹, Glenn Terry², and Mac Davis¹

¹Minicircle, Inc.

²Global Alliance of Regenerative Medicine

Correspondence concerning this article should be addressed to Mac Davis.

Email: mac@minicircle.io

Abstract

Plasmids as a gene delivery method have been largely underused by the healthcare industry, despite the fact that they offer a lower risk and less-costly alternative to viral vectors. Because of the focus on viral vector development, along with the fact that gene therapy is a relatively young field, plasmid duration of action in the human body is so far poorly understood. The goal of this study was to investigate the temporal profile of plasmid-delivered follistatin longitudinally. This objective was achieved by measuring serum follistatin levels in a group of adult humans before and after a plasmid-based follistatin (FST 344) injection. Participants were measured at baseline, 3-month, and 1-year marks. Analyses showed a substantial increase in follistatin levels from an average of 8.75 ng/mL at baseline to 22.50 ng/mL at 3 months. These elevated levels eventually decreased to an average of 21.10 ng/mL at the 1-year mark. No adverse effects were reported aside from temporary mild irritation at the injection site. Overall, these findings add to the evidence that plasmids are a viable and potentially long-lasting method for delivering genes. The decrease in concentration from 3 months to 1 year suggests that the initial elevated FST concentration is not permanent, which would be a safety advantage in the case of any unexpected adverse events. This study is the first to demonstrate that plasmid-delivered follistatin can be efficacious and long-lasting in humans.

Key words: follistatin, plasmid, viral vector, gene therapy, epigenetic age, healthy longevity

Introduction

Although nonviral plasmids offer a potentially safer and cheaper alternative to viral vectors for DNA delivery (Hardee et al., 2017), debates over their efficacy have limited their widespread adoption and development. Historically, there has been discourse over their effectiveness at penetrating the cell, as well as how long they remain effective. While research has led to the understanding that plasmids can be effective when combined with a transfection agent, questions about duration of action have persisted due to the (relative) dearth of longitudinal studies on plasmid gene therapy. The goal of the present study was to understand the temporal profile of plasmid-delivery follistatin in adult humans longitudinally.

Nonviral plasmid gene therapies have been viewed as safe for decades. However, their lesser transfection efficiency and duration compared to viral vector gene therapies has led to them being underdeveloped. Today, most DNA therapies and vaccines use viral vectors despite known risks such as innate immunity and mutagenesis, both of which, though rare, can be fatal or cause severe health complications.

The goal of healthcare and science is to increase health outcomes while decreasing risks, so if plasmids offer a lower risk and more accessible (cheaper) alternative to viral vectors, they should be investigated. Efforts have been made to improve plasmid duration, with notable successes including minicircles (Darquet et al., 1997; Chen et al., 2003), scaffold/matrix-attached region- (S/MAR-) containing episomal plasmids (Jenke et al., 2002), and Mini-Intronic Plasmids (MIPs) (Lu et al., 2013). A number of these plasmids and/or their derivatives have lasted at least 6-8 months in animal models, but research beyond this time window is lacking. In humans, at least one study showed a plasmid lasting over three months (Maulik et al., 2009; see Table 1).

Table 1
Longest plasmid duration results in animals and humans

DURATION	PLASMID	GENE	TISSUE	ORGANISM	REFERENCE
8+ mo.	pFAR4	eGFP	Liver	Mouse	Manta et al., 2017
7 mo.	MIP	hFIX	Liver	Mouse	Lu et al., 2013
6 mo.	SMAR	Luc.	Liver	Mouse	Argyros et al., 2008
150-160 d	pEPIt0	Luc.	Sk. Musc.	Mouse	Neto et al., 2015
3+ mo.	pCOR	FGF1	Systemic (IM)	Human	Maulik et al., 2009

The discussion so far about viral and nonviral delivery systems is important because gene therapy is on the verge of a rapid acceleration in its development. Given that gene therapy is a relatively new field (with the human genome having been sequenced in the early 2000's), it will be especially important moving forward to continue to improve health intervention risk profiles while characterizing efficacy and duration. In the present study, follistatin was chosen as a gene therapy to test because its mechanism of action is well-understood (Hedger et al., 2011) and

because of its potential to have profound effects on lifespan and healthy longevity (Jaijyan et al., 2022). Follistatin gene therapy has so far been researched as a treatment for muscular dystrophy due to its ability to increase and regulate muscle mass and strength (Al-Zaidy et al., 2015). Meanwhile, preclinical studies in nonhuman primates also suggest that it has globally useful effects for enhancing muscle composition, even in the absence of a disease (Kota et al., 2009).

Overall, the field's previous shift away from plasmid development may not be warranted if the main limitations of plasmids can be addressed (i.e., transfection efficiency and duration). Transfection efficiency can generally be managed by using a transfection agent. Additionally, advances in science, such as minicircle DNA, support increased duration. The research presented here builds on previous findings from our laboratory, which showed elevated serum follistatin levels three months after a plasmid-delivered follistatin injection. The goal of the present study was to investigate the temporal profile of serum follistatin longitudinally in response to a gene therapy intervention.

Methods

Overview

A formulation containing FST 344 isoform was prepared and administered. Serum follistatin was measured prior to administration, 3 months post-administration, and 1 year post-administration in a final cohort of 27 healthy adult volunteers.

Participants

To be included in the study, participants had to be adults over the age of 23 without any genetic disorders or autoimmune diseases. Participants were excluded if they were unable to travel, were pregnant or lactating, or had a genetic disorder or autoimmune disease.

This study was registered at clinicaltrials.gov as #NCT06411366. Ethics approval was obtained from the Global Alliance for Regenerative Medicine (GARM) IRB committee. Written informed consent was obtained from all participants. Participants were volunteers who did not receive any financial compensation. Participants were asked to pay for their own lab work and travel but financial support was provided to participants in need. The test treatment was provided at no cost to participants.

Plasmid Construct

A plasmid was constructed delivering the 344 amino acid secreted isoform of the human follistatin gene. Transgene expression was driven by a CMV promoter.

Follistatin isoform: FST 344 was used to increase circulating FST 315 based on specific characteristics of FST 344 as well as prior research groups' results¹². FST is initially produced in two isoforms, FST 317 and FST 34422 (see Fig. S1). Both are secreted then subsequently

cleaved from their secretory signal peptide, resulting in FST 288 and FST 315, respectively. FST 344 and FST 315 contain a C-terminal acidic region that prevents FST 315 from binding to the plasma membrane's extracellular surface, subsequently allowing FST 315 to be released into circulation. FST 317 and FST 288 lack this C-terminal feature, therefore FST 288 is not the major circulating form of FST.

FST is a secreted protein, meaning it is made in one cell, exported to the cell surface, cleaved of its signal peptide, and released into systemic circulation via the blood²⁰. This means we could transfect a small number of cells and have them become export factories for FST. Thus, we injected the plasmid subcutaneously to transfect a small number of adipocytes capable of subsequently creating systemic effects (see Fig. 1).

Vector Purification and Characterization: The sequence was verified by third party synthesis post-oligo ligation via m-13 reverse and forward primers as well as next-generation sequencing by GeneWiz. Endotoxin concentration was determined to conform to FDA Guidance for Industry recommendations via kinetic LAL assay performed by third-party CRO Charles River.

Transformation Technique: Transfection was achieved by means of 40 kDa linear polyethyleneimine (Polysciences catalog no. 24765- 1) coadministration with plasmid. This relatively low molecular weight PEI was chosen to reduce cytotoxicity (possible ranges are 25-800 kDa; xx cite). A 4:1 ratio of transfection agent to plasmid was used as suggested by the manufacturer, Polysciences.

Procedure

The follistatin gene therapy was injected at the GARM clinic in Próspera ZEDE. Two sterile vials, one containing plasmid and one containing PEI, were mixed and incubated at room temperature for 30 minutes prior to administration. After incubation, 400 μ l of saline solution containing 50 μ g of plasmid was injected into subjects' abdominal subcutaneous fat. The primary physician observed patients for 15 minutes post-injection in case of allergic reactions or other complications.

Serum follistatin was measured prior to administration, three months post-administration, and one year post-administration. The FST 344 concentration was determined using the Invitrogen Follistatin (FST) Human ELISA Kit. This assay kit is a colorimetric sandwich-type ELISA provided by ThermoFisher, catalog #EHFST.

Participant inclusion and data selection

Participants were included in this analysis if they had serum follistatin measurements at baseline (month 0) and at least one post-anniversary timepoint (either 1 year or 2 years post-

injection). For year 1 or year 2 follow-up, the follistatin concentration used in the analysis was selected as follows:

1. Samples noted by the ELISA laboratory technician as problematic (e.g., turbid or hemolyzed) were excluded.
2. If a single blood draw was tested on multiple dates, only the chronologically earliest measurement was used.
3. Of the remaining eligible results within a ± 6 month window around the post-injection anniversary, the measurement closest in time to the anniversary was selected to represent that timepoint (year 1 or year 2).
4. One participant was excluded from the year 2 analysis due to receiving an unrelated experimental treatment that transiently altered follistatin expression, as evidenced by a characteristic suppression pattern in post-treatment follow-up samples.

Analyses

Given the presence of skewed distributions and outliers at multiple timepoints (including one statistical extreme at month 0), normality assumptions for parametric repeated-measures methods were not met. Shapiro–Wilk tests indicated departures from normality at baseline ($p = .008$), 3 months ($p < .001$), and 2 years ($p = .012$), with only Year 1 data consistent with normality ($p = .296$). Q–Q plots further confirmed non-normality for most timepoints.

Because of these violations and the small sample sizes at later timepoints, we used paired Wilcoxon signed-rank tests to compare each follow-up timepoint to baseline. This non-parametric method is robust to skew and outliers, while preserving power in small, paired datasets. Three pre-specified comparisons (baseline $\rightarrow$ 3 months, baseline $\rightarrow$ year 1, baseline $\rightarrow$ year 2) were tested, with Holm adjustment for multiple comparisons. Effect sizes (rank-biserial correlation) and 95% confidence intervals were calculated for each contrast.

Results

Median serum follistatin increased markedly from baseline at all follow-up timepoints (Figure 1). Baseline median was 8.65 ng/mL (IQR = 2.36, $n = 22$). Levels rose to 22.50 ng/mL (IQR = 13.50, $n = 19$) at 3 months, 21.10 ng/mL (IQR = 19.70, $n = 12$) at 1 year, and 11.80 ng/mL (IQR = 12.40, $n = 15$) at 2 years.

Paired Wilcoxon signed-rank tests comparing each follow-up to baseline indicated large effect sizes for all three pre-specified contrasts (rank-biserial $r = 0.88$ [95% CI 0.88–0.88] at 3 months; 0.82 [0.55–0.89] at 1 year; 0.79 [0.57–0.88] at 2 years). All comparisons remained statistically significant after Holm adjustment (adjusted $p < .001$, .005, and .005, respectively).

No adverse events beyond temporary mild irritation at the injection site were reported during follow-up.

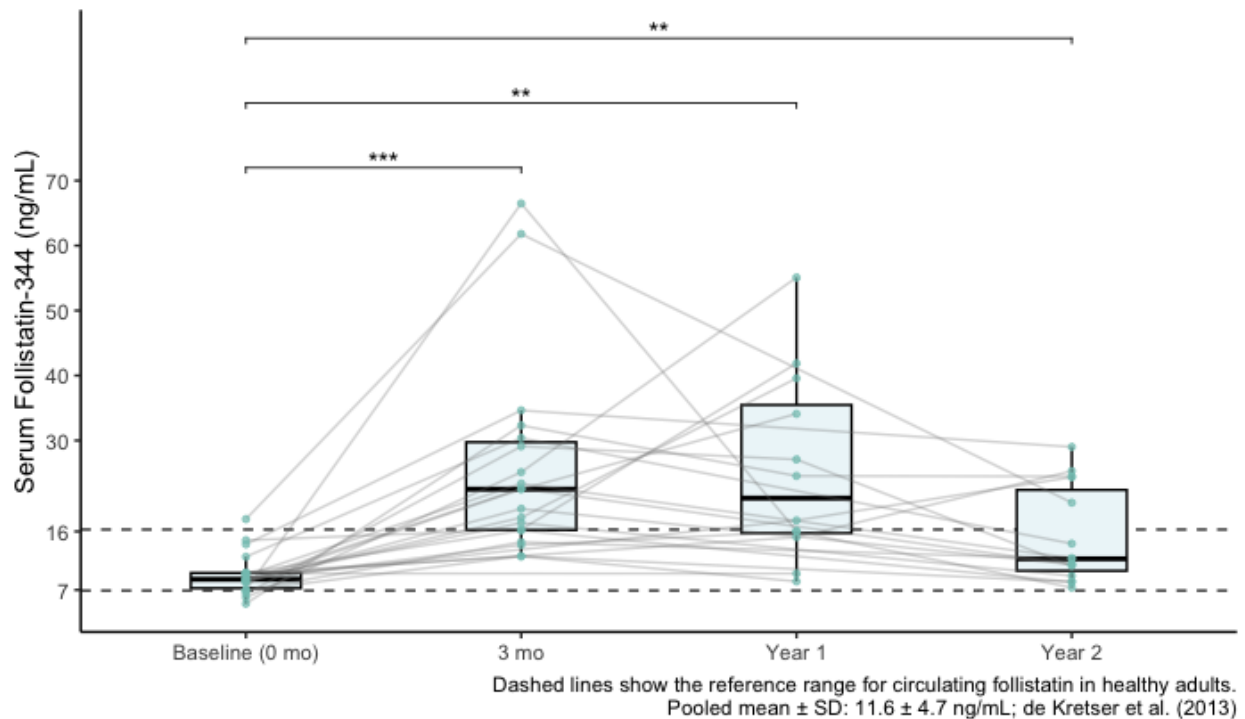


Figure 1

Serum follistatin levels across time

Serum follistatin-344 concentrations at baseline and follow-up timepoints after plasmid-based gene therapy. Boxplots show medians and interquartile ranges; thin gray lines connect paired values for individual participants. Dashed lines indicate the reference range for circulating follistatin in healthy adults (pooled mean $\pm$ SD: 12.6 $\pm$ 4.5 ng/mL; de Kretser et al., 2013). Asterisks denote significance from baseline using paired Wilcoxon signed-rank tests with Holm-adjusted p-values: $p < .05$ (*), $p < .01$ (**), $p < .001$ (***)

Discussion

This study aimed to answer two main questions: 1) Does plasmid delivery follistatin work? 2) What is the temporal profile of serum follistatin in response to this gene therapy longitudinally?

Answering the first question requires us to consider how biological delivery systems have been understood historically. The adoption of viral vectors took place largely because viruses are exceptional at delivering genes to cells. Meanwhile, plasmids have been generally underused in healthcare, originally due to concerns about their effectiveness and ability to transfect cells. This led to a decrease in efforts to further develop plasmids so that they could transfect reliably, and instead there was a shift in focus toward viral vectors. However, the risks of viral vectors, such

as insertional mutagenesis and innate immunity, remain a relevant concern. Strict regulation has for the most part prevented these risks from reaching the general public, but multiple fatalities have occurred in the past decade where a viral-based drug therapy was ruled as a likely player.

The scientific community's responsibility is to continue to improve health interventions while decreasing risks. If plasmids can offer a lower risk profile than viral vectors, it is worth exploring their viability as a gene therapy delivery system. The findings from our study showed that a plasmid-based follistatin injection resulted in increased follistatin levels 3 months later, and that these levels remained elevated above baseline 1 year after injection. These findings provide evidence in favor of plasmids being a viable method for delivering gene therapies. This is important to consider given that the main reason plasmids have been underused in healthcare was originally due to concerns about their effectiveness.

The second goal of this study was to characterize the temporal profile of plasmid-delivered follistatin over an extended follow-up. Serum follistatin remained significantly elevated compared to baseline at both 1 year and 2 years after injection. Median levels peaked at 3 months, declined slightly by 1 year, and showed further regression toward baseline by 2 years. This pattern suggests that the initial spike observed at 3 months is not permanent, which could be a safety advantage in the event of adverse effects. Nevertheless, the persistence of elevated levels in most participants up to 2 years post-treatment ($n = 15$) indicates a substantial and prolonged duration of action. Future studies should investigate the underlying mechanisms of individual variability and assess the temporal profile beyond 2 years.

It should also be noted that the only adverse effect reported was temporary irritation at the injection site. No severe or moderate adverse events have been reported. Participants have been asked to contact the research team if any adverse events that are likely related to the gene therapy occur in the future.

In summary, plasmids offer a safer, more cost-effective alternative to viral methods for DNA delivery, and it would be beneficial to the scientific community to continue developing them. The healthcare industry has largely underutilized plasmids due to concerns about efficaciousness and duration of action. However, our study found that plasmid-based follistatin was successful at increasing serum follistatin levels for as long as 1 year after administration, which provides evidence in favor of plasmids being a viable gene delivery method.

References

- Al-Zaidy SA, Sahenk Z, Rodino-Klapac LR, Kaspar B, Mendell JR. Follistatin Gene Therapy Improves Ambulation in Becker Muscular Dystrophy. *J Neuromuscul Dis.* 2015 Sep 2;2(3):185-192. doi: 10.3233/JND-150083. PMID: 27858738; PMCID: PMC5240576.
- Argyros O, Wong SP, Niceta M, Waddington SN, Howe SJ, Coutelle C, Miller AD, Harbottle RP. Persistent episomal transgene expression in liver following delivery of a scaffold/matrix attachment region containing non-viral vector. *Gene Ther.* 2008 Dec;15(24):1593-605. doi: 10.1038/gt.2008.113. Epub 2008 Jul 17. PMID: 18633447.
- Chen ZY, He CY, Ehrhardt A, Kay MA. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther.* 2003 Sep;8(3):495-500. doi: 10.1016/s1525-0016(03)00168-0. PMID: 12946323.
- Darquet AM, Cameron B, Wils P, Scherman D, Crouzet J. A new DNA vehicle for nonviral gene delivery: supercoiled minicircle. *Gene Ther.* 1997 Dec;4(12):1341-9. doi: 10.1038/sj.gt.3300540. PMID: 9472558.
- de Kretser DM, Bensley JG, Pettilä V, Linko R, Hedger MP, Hayward S, Allan CA, McLachlan RI, Ludlow H, Phillips DJ, et al. Serum activin A and B levels predict outcome in patients with acute respiratory failure: a prospective cohort study. *Crit Care.* 2013;17:R263. doi:10.1186/cc13093. PMID:24172607; PMCID: PMC4057391
- Haase R, Argyros O, Wong SP, Harbottle RP, Lipps HJ, Ogris M, Magnusson T, Vizoso Pinto MG, Haas J, Baiker A. pEPito: a significantly improved non-viral episomal expression vector for mammalian cells. *BMC Biotechnol.* 2010 Mar 15;10:20. doi: 10.1186/1472-6750-10-20. PMID: 20230618; PMCID: PMC2847955.
- Hardee CL, Arévalo-Soliz LM, Hornstein BD, Zechiedrich L. Advances in Non-Viral DNA Vectors for Gene Therapy. *Genes (Basel).* 2017 Feb 10;8(2):65. doi: 10.3390/genes8020065. PMID: 28208635; PMCID: PMC5333054.
- Hedger MP, Winnall WR, Phillips DJ, de Kretser DM. The regulation and functions of activin and follistatin in inflammation and immunity. *Vitam Horm.* 2011;85:255-97. doi: 10.1016/B978-0-12-385961-7.00013-5. PMID: 21353885.
- Jaijyan DK, Selariu A, Cruz-Cosme R, Tong M, Yang S, Stefa A, Kekich D, Sadoshima J, Herbig U, Tang Q, Church G, Parrish EL, Zhu H. New intranasal and injectable gene therapy for healthy life extension. *Proc Natl Acad Sci U S A.* 2022 May 17;119(20):e2121499119. doi: 10.1073/pnas.2121499119. Epub 2022 May 10. Erratum in: *Proc Natl Acad Sci U S A.* 2022 Aug 30;119(35):e2212836119; 2023 Aug 8;120(32):e2311483120. PMID: 35537048; PMCID: PMC9171804.
- Jenke BH, Fetzer CP, Stehle IM, Jönsson F, Fackelmayer FO, Conradt H, Bode J, Lipps HJ. An episomally replicating vector binds to the nuclear matrix protein SAF-A in vivo. *EMBO Rep.*

2002 Apr;3(4):349-54. doi: 10.1093/embo-reports/kvf070. Epub 2002 Mar 15. PMID: 11897664; PMCID: PMC1084058.

Kota J, Handy CR, Haidet AM, Montgomery CL, Eagle A, Rodino-Klapac LR, Tucker D, Shilling CJ, Therlfall WR, Walker CM, Weisbrode SE, Janssen PM, Clark KR, Sahenk Z, Mendell JR, Kaspar BK. Follistatin gene delivery enhances muscle growth and strength in nonhuman primates. *Sci Transl Med*. 2009 Nov 11;1(6):6ra15. doi: 10.1126/scitranslmed.3000112. PMID: 20368179; PMCID: PMC2852878.

Lu J, Zhang F, Kay MA. A mini-intronic plasmid (MIP): a novel robust transgene expression vector in vivo and in vitro. *Mol Ther*. 2013 May;21(5):954-63. doi: 10.1038/mt.2013.33. Epub 2013 Mar 5. PMID: 23459514; PMCID: PMC3666631.

Manta S, Renault G, Delalande A, Couture O, Lagoutte I, Seguin J, Lager F, Houzé P, Midoux P, Bessodes M, Scherman D, Bureau MF, Marie C, Pichon C, Mignet N. Cationic microbubbles and antibiotic-free miniplasmid for sustained ultrasound-mediated transgene expression in liver. *J Control Release*. 2017 Sep 28;262:170-181. doi: 10.1016/j.jconrel.2017.07.015. Epub 2017 Jul 11. PMID: 28710005.

Maulik N. NV1FGF, a pCOR plasmid-based angiogenic gene therapy for the treatment of intermittent claudication and critical limb ischemia. *Curr Opin Investig Drugs*. 2009 Mar;10(3):259-68. PMID: 19333884.

Neto MF, Letteri R, Chan-Seng D, Emrick T, Figueiredo ML. Sonodelivery Facilitates Sustained Luciferase Expression from an Episomal Vector in Skeletal Muscle. *Materials (Basel)*. 2015 Jul 1;8(7):4608-4617. doi: 10.3390/ma8074608. Epub 2015 Jul 22. PMID: 26834950; PMCID: PMC4730882.

Yokoo T, Kamimura K, Abe H, Kobayashi Y, Kanefuji T, Ogawa K, Goto R, Oda M, Suda T, Terai S. Liver-targeted hydrodynamic gene therapy: Recent advances in the technique. *World J Gastroenterol*. 2016 Oct 28;22(40):8862-8868. doi: 10.3748/wjg.v22.i40.8862. PMID: 27833377; PMCID: PMC5083791.