Infectivity and Expression in Mice of an Anelloviral Vector Produced by *In Vitro* Assembly

Isabella Gold, Riana Pozsgai, Erik Hansen, Timsi Rao, Andrew Keezer, Yue Zhang, Madeline Schanen, Eli Miller, Stephanie Thurmond, Nidhi Mukund Acharekar, Hyun Jung Jun, Geoffrey Parsons, Chris Wright, Ashley Mackey
Ring Therapeutics, Cambridge, MA, USA

Anellovirus Background

The anelloviridae family comprises a diverse spectrum of commensal viruses found throughout the human body. The abundance of anelloviruses in various tissues, in addition to their lack of disease associations, were inspirations to develop an Anelloviral vector to deliver a therapeutic nucleic acid payload[1].

The commensal nature of our Anelloviral vectors (ANV) manifests in their potential for minimal immunogenicity, and candidacy as commercial gene delivery vectors, with potential for redosing[2-4], unlike AAV. One of the largest drawbacks of current gene therapies is the price, with a cost of anywhere from $800,000 to $3 million per treatment[5]. The development of a new, cell-free, *in vitro* assembly platform called AnelloBricks® has the potential to cut production costs dramatically and allow for a modular approach to vector engineerability.

We have demonstrated that the AnelloBricks® platform is functional in both *in vitro* and *in vivo* models, by assessing DNA infectivity, mRNA transcripts and protein expression of a payload (eGFP). Platform capabilities are continually enhanced by ongoing changes in the manufacturing and production process. These *in vitro* and *in vivo* experiments aid in the enhancement of the *in vitro* assembly process, which has the potential to transform the field of gene therapy.

Modular AnelloBricks® Production Platform

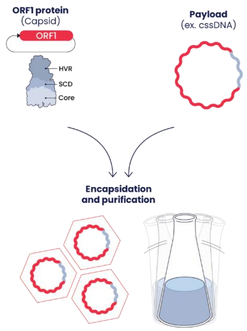


Figure 1: **AnelloBricks® Platform**

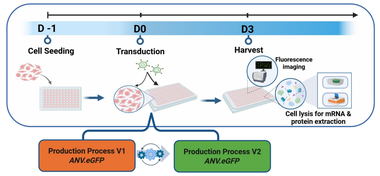
AnelloBricks® are composed of recombinant anellovirus ORF-1 capsid protein and nucleic acid payload. Both components are produced separately and then combined at the optimal capsid:payload ratio to form symmetrical virus-like particles. This method of anelloviral vector production is amenable to capsid and payload engineering to optimize transduction efficiency.

In Vitro and *In Vivo* Validation of AnelloBricks® Production Process Improvements

AnelloVector® (ANV) Transduction of HEK293TT Cells

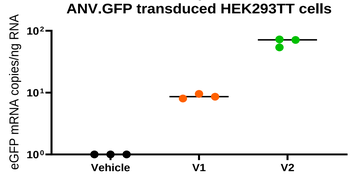
***Figure 2*: Transduction efficiency of AnelloVector® (ANV.eGFP) in HEK293TT cells is improved by optimization of the AnelloBricks® process.** The objective of these experiments was to assess transduction efficiency of our AnelloVectors® (ANV.eGFP) *in vitro* and to evaluate any improvements of our vector in HEK293TT cells between production runs. Cells were transduced with either vehicle (represented in black throughout), Production Process V1 material (initial manufacturing process, represented in orange throughout), or Production Process V2 material (current manufacturing process, represented in green throughout).

(A)



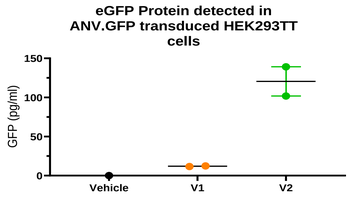
HEK293TT cells were transduced with ANV.eGFP. 3 days post-transduction, fluorescent images were captured, and cells were lysed for either mRNA extraction followed by eGFP analysis by RT-ddPCR or protein extraction for eGFP quantification using the Ella™ Automated ELISA platform (Bio-Techne).

(B)



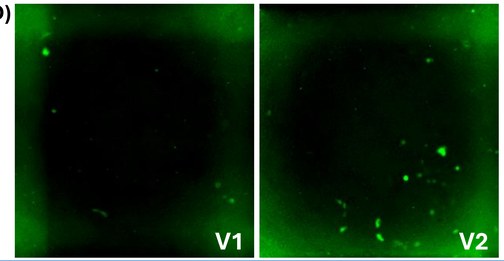
10-fold increase in eGFP mRNA transcripts observed with Production Process V2. Biological replicates are represented on the graph as data points, with 3 transduced wells analyzed per treatment group. eGFP mRNA transcripts were quantified by RT-ddPCR.

(C)



10X increase in eGFP protein observed in Production Process V2. Biological replicates are represented on the graph as data points, with 2 transduced wells analyzed per treatment group. eGFP signal (pg/mL) was quantified in cell lysate by Ella™ Automated ELISA platform (Bio-Techne).

(D)



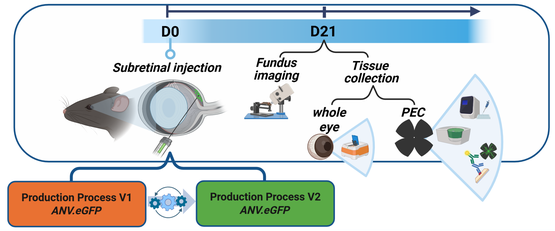
HEK293TT cells express endogenous eGFP, observed by fluorescent imaging. HEK2923TT cells transduced with ANV.eGFP at an MOI of 5K were imaged at a 10X magnification using the Cytation 5 with a eGFP filter (469nm/525nm) to detect eGFP expression. Representative images shown here illustrate minimal signal found in the Production Process V1 treated cells and increased eGFP signal in the Production Process V2 treated cells.

AnelloVector® (ANV) Transduction of Rodent Ocular Tissue

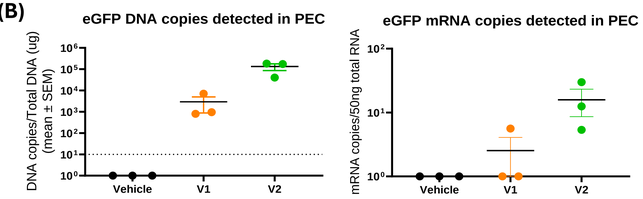
***Figure 3*: Transduction efficiency of AnelloVector® (ANV.eGFP) in the rodent eye is improved by optimization of the AnelloBricks® process.** AnelloVectors® (ANV.eGFP) were administered subretinally (SR) into rodent eyes to assess production method improvements. Eyes were injected with either vehicle (represented in black throughout), Production Process V1 material (initial manufacturing process, represented in orange throughout), or Production Process V2 material (current manufacturing process, represented in green throughout).

(A)

ANV.eGFP was administered bilaterally via subretinal injection into C57Bl/6J mouse eyes. 21 days post-dose, eyes were imaged via fundus imaging and harvested. The posterior eye cup (PEC) was evaluated for eGFP expression and genome copies by qPCR, RT-ddPCR and IHC. Whole eyes were collected for eGFP quantification by the Ella™ Automated ELISA platform.



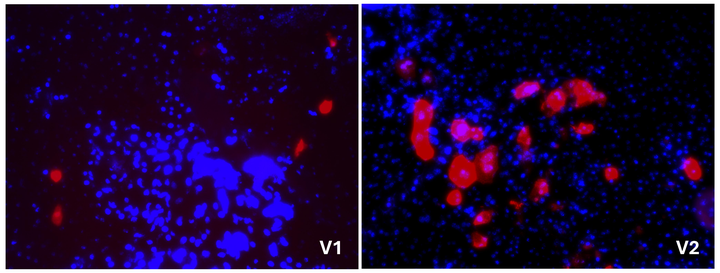
(B)



10-fold increase in eGFP genome copies and mRNA transcripts observed in Production Process V2 as compared to Production Process V1. Individual eyes are represented on the graph as data points, with 3 eyes analyzed per treatment group. eGFP genome copies were detected in the PEC by qPCR. eGFP mRNA transcripts were detected in the PEC by RT-ddPCR.

(C)

RPE cells expressing eGFP were detected by IHC in PEC wholemounts, as shown in representative images. After SR injection, the PEC's were fixed with 4% PFA and imaged, where endogenous eGFP expression was observed. Subsequently, IHC was performed using a eGFP monoclonal antibody. RPE transduced cell counts increased approximately 2X from Process V1 to V2. eGFP protein was also detected using the Ella™ Automated ELISA platform (Bio-techne); data not shown. (20X magnification)



Conclusion

- AnelloVectors® produced from the AnelloBricks® platform can functionally deliver a cssDNA payload and result in confirmed mRNA and protein expression in both *in vitro* and *in vivo* models.
- Improvements to Ring's production process (V1 vs V2) have increased transduction efficiency by approximately 10-fold in both *in vitro* and *in vivo* models.
- *Path Forward*→ Development of engineered ORF-1 capsid and/or engineered payload to further improve ANV infectivity and expression across various tissues and cell lines.

AnelloBricks® production poster
Tues, May 13, 6:00-7:30 PM, Poster Hall I2, Poster 867
Purified Anelloviral Capsid Proteins can Bind and Encapsidate Different Nucleic Acid Payloads into AnelloVectors® for Gene Delivery.

Oral presentation
Thurs, May 15, 4:45-5:00 PM, New Orleans Theater B
AnelloBricks: Development of a Scalable, Low-Cost, In-Vitro Assembled Anellovirus-Derived Platform for Gene Therapy Applications.

Acknowledgements
We would like to thank our internal teams at Ring Therapeutics for their collaboration, guidance, and support at every stage of the project.

References:
1. Prince C, et al., A novel functional gene delivery platform based on a commensal human anellovirus demonstrates transduction in multiple tissue types. *bioRxiv* 2024
2. Arze CA, et al., Global genome analysis reveals a vast and dynamic anellovirus landscape within the human virome. *Cell Host Microbe.* 2021 Aug 11;**29**(8):1305-1315.e6. doi: 10.1016/j.chom.2021.07.001.
3. Liou S, et al., S. Anellovirus structure reveals a mechanism for immune evasion. *bioRxiv* 2022
4. Nawandar DM, et al., Human anelloviruses produced by recombinant expression of synthetic genomes. *bioRxiv* 2022
5. Buntz B. How price, safety, and efficacy shape the cell and gene therapy landscape. Drug Discovery Trends. Published May 10, 2024.