Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2021 April ; 41(4): e240–e242. doi:10.1161/ATVBAHA.121.315965.

Response by Dichek to Letter Regarding Article, "Jugular Vein Injection of HighTiter Lentiviral Vectors Does Not Transduce the Aorta"

David A. Dichek

Division of Cardiology, Department of Medicine, University of Washington, Seattle.

To the Editor:

We understand and empathize with Dr. Redondo's disappointment with our report.[1] We share his disappointment because we so much wanted this to work! Regarding specific critiques, we reaffirm that the titers of the lentivirus (LV) preparations that we used are within the range of those reported by Dr. Redondo and his collaborators, who also reported success with an LV preparation of 1×10^8 transducing units/mL,[2] 90% below the 1×10^9 transducing units/mL that Dr. Redondo recently suggested as essential,[3] and well below the titers of the LV preparations that we used. All studies, including ours, have limitations. However, positive controls included in our report confirm our ability to detect high-level GFP expression in mouse tissue if present, our ability to extract and detect small amounts of LV DNA if present in mouse arteries transduced either in vitro (aortic segment) or in vivo (several carotid arteries), and our ability to inject sufficient intravascular LV to achieve in vivo gene transfer to tissues susceptible to LV transduction (liver and spleen) with 100% reproducibility. Our standard curves confirm our ability to detect the GFP DNA sequence with high sensitivity, even if it is contained in a small DNA fragment such as a plasmid. Fragmentation of genomic DNA in aortic extracts cannot explain our negative results.

Dr. Redondo criticizes our immunohistochemistry as inconsistent with our LV genome quantitation and lacking in sensitivity. Importantly, our immunohistochemistry was not designed to quantify LV genomes, if that could even be achieved by immunostaining. There are simple explanations for why results of LV genome quantitation and immunostaining of GFP in the same tissue would not match. A single GFP-expressing cell may contain several LV genomes. Alternatively, an unstained cell may contain LV genomes that are not expressing GFP, for example, because they are integrated in a silent area of the genome. As concerns sensitivity, our staining technique can clearly detect low-level LV transduction as shown by positive staining of spleen and liver (0.8 and 0.1 LV genomes/cell, respectively) and of right carotid arteries (~1 LV genome/cell). Supporting the specificity of our immunostaining, it is expected that GFP staining would be confined to the outer surfaces of the in vivo transduced carotids and the ex vivo transduced aorta, and that is exactly where we find it. Finally, Dr. Redondo misrepresents the methods we used to generate the ex vivo positive controls. The aortic segment used for DNA extraction (Figure 1B) was transduced at 5×10^7 transducing units/mL, whereas the segment used for immunostaining (Figure 2B) was transduced at 1×10^7 transducing units/mL. It is unsurprising that GFP staining of the aortic

segment is less abundant or intense than might be expected in tissue with ~12 LV genomes per cell.

It is curious that Dr. Redondo questions our technical experience and training. Our laboratory has worked on vascular cell gene transfer for over 30 years.[4] We have paid particular attention to quantitative aspects of arterial gene transfer[5] and to defining the cell-type specificity of arterial gene transfer.[6] We optimized methods for in vivo gene transfer to the artery wall,[7] and we defined the role of endothelial barrier function in determining the distribution of viral vectors and the location of transduced cells in the artery wall.[8] We were the first group to report in vivo transduction of mouse arteries.[9] All of this work has withstood the test of time. The operator in the present study (LB) is a skilled microsurgeon who has performed scores of arteriotomies and vector injections in rabbits.[10, 11]

If, as suggested by Dr. Redondo, our negative results are due to insufficient technical experience and training, this will become apparent when multiple laboratories independently report convincing molecular evidence of efficient intravenous LV-mediated gene transfer to the aorta. This evidence would be expected to include immunohistochemical, histochemical, and fluorescence microscopy detection of cell-localized expression of multiple LV-expressed reporter genes.[12–14] It would be expected that these methods would not reveal—as portrayed in Dr. Redondo's publications—diffusely positive staining of ~100% of aortic mural cells and surrounding tissues.[15, 16] Rather, it would be expected that some cells would be distinctly positive for reporter gene expression and others would be negative, as reported by others in efficiently LV-transduced mouse tissues.[12–14] Evidence of successful aortic transduction would also need to include robust detection of LV DNA, in an amount sufficient to explain phenotypic effects. To our knowledge, detection of aortic LV DNA in LV-injected mice has never been reported by any group. As explained in more detail in our report,[1] presence of LV DNA is a critical factor in confirming LV-mediated gene transfer. Detection of aortic LV DNA is not reported in any of Dr. Redondo's own or collaborative publications[2, 3, 15–17] nor is it reported in any of the other 4 papers he cites as supportive of his work.[18–21] None of these papers provide convincing evidence of cell-localized recombinant gene expression in the aorta. Indeed one of these papers does not even include a control group for detection of aortic GFP fluorescence (Xu et al; Fig. S2A).[20] All 4 papers use tail vein injections of low-titer LV preparations (1×10^7–2×10^8 transducing units/mL), an injection site and titers that Dr. Redondo has associated with failure to achieve efficient aortic transduction.[3] Rather than being supportive of Dr. Redondo's claims, these papers present yet another challenge to his conceptualization of how intravenous LV-mediated aortic gene transfer might work.

It is at least 10 years since in vivo LV-mediated aortic transduction was first reported.[15] In 2021, there are still only a few reports of success. Most of these reports are co-authored by Dr. Redondo,[2, 3, 15–17] and all of these reports lack critical data that would support efficacy. We suggest a pathway forward for Dr. Redondo, including steps that could help him to establish broad confidence in his results. (1) Publish methods for LV injection and detection in a publicly accessible format such as the *Journal of Visualized Experiments*. We have reported our own detailed vascular gene transfer methods in this format.[11, 22] (2) Measure LV DNA in aortas, livers, spleens, and bone marrow of LV-infused mice and report his

results. If abundant aortic LV DNA is found, we would be glad to learn how we missed it. If abundant aortic LV DNA is not found, Occam's razor says that the aorta is not transduced and that diffuse GFP immunostaining suggesting otherwise[15, 16] is a falsely positive indicator of aortic transduction. (3) Measure LV DNA in tissue adjacent to the jugular vein injection site. This approach is likely to provide a far more sensitive assessment of local LV release than is provided by the acute visual assessment of Evans blue dye leakage that Dr. Redondo suggests in his letter. (4) Provide a plausible biological explanation (as well as supportive experimental data) for how injection of 10^7–10^8 LV transducing units into a mouse jugular vein could yield ~100% transduction of aortic cells. This level of aortic cell transduction is implausible because it conflicts with numerous quantitative studies from leading gene-therapy groups showing that the mouse liver (~3.5×10^8 cells),[23] spleen (~2×10^8 cells),[24] and bone marrow (~5×10^8 cells)[25] are all highly transduced by intravenously injected LV vectors whereas organs with large amounts of arterial and other vascular tissue (lung, heart, kidney) are essentially not transduced.[12–14, 26–28] (5) Explain why injection of LV by his collaborators seems to transduce only the aortic luminal endothelium (de Yebenes et al.,[17] Figure 5B, in which tissue and antibody controls are also absent), whereas LV injections in his own laboratory with the same LV pseudotype and backbone seem to transduce 100% of cells throughout the full thickness of the aortic wall.[15, 16] (6) Finally, we encourage Dr. Redondo to accept my invitation, offered on 12/11/20, to engage in precommitment that would lead to a registered report[29] of results of experiments aimed at reproducing highly efficient aortic transduction after intravenous injection of LV vectors. Following these 6 suggestions would allow the field to move beyond recrimination and instead refocus community attention on experimental data and on methods that are both detailed and easy to reproduce.

References

1. Bi L, Wacker BK, Stamatikos A, Sethuraman M, Komandur K, Dichek DA. Jugular vein injection of high-titer lentiviral vectors does not transduce the aorta. Arterioscler. Thromb. Vasc. Biol. 2020:ATVBAHA120315125
2. Martin-Alonso M, Garcia-Redondo AB, Guo D, et al.Deficiency of mmp17/mt4-mmp proteolytic activity predisposes to aortic aneurysm in mice. Circ. Res. 2015;117:e13–26 [PubMed: 25963716]
3. Alfranca A, Campanero MR, Redondo JM. New methods for disease modeling using lentiviral vectors. Trends Mol Med. 2018;24:825–837 [PubMed: 30213701]
4. Dichek DA, Neville RF, Zwiebel JA, Freeman SM, Leon MB, Anderson WF. Seeding of intravascular stents with genetically engineered endothelial cells. Circulation. 1989;80:1347–1353 [PubMed: 2509105]
5. Flugelman MY, Jaklitsch MT, Newman KD, Casscells W, Bratthauer GL, Dichek DA. Low level in vivo gene transfer into the arterial wall through a perforated balloon catheter. Circulation. 1992;85:1110–1117 [PubMed: 1537109]
6. Schulick AH, Dong G, Newman KD, Virmani R, Dichek DA. Endothelium-specific in vivo gene transfer. Circ. Res. 1995;77:475–485 [PubMed: 7641320]
7. Schulick AH, Newman KD, Virmani R, Dichek DA. In vivo gene transfer into injured carotid arteries. Optimization and evaluation of acute toxicity. Circulation. 1995;91:2407–2414 [PubMed: 7729028]
8. Rome JJ, Shayani V, Flugelman MY, Newman KD, Farb A, Virmani R, Dichek DA. Anatomic barriers influence the distribution of in vivo gene transfer into the arterial wall. Modeling with microscopic tracer particles and verification with a recombinant adenoviral vector. Arterioscler. Thromb. 1994;14:148–161 [PubMed: 8274471]

9. Vassalli G, Agah R, Qiao R, Aguilar C, Dichek DA. A mouse model of arterial gene transfer. Antigen-specific immunity is a minor determinant of the early loss of adenovirus-mediated transgene expression. Circ. Res. 1999;85:e25–e32 [PubMed: 10532959]
10. Bi L, Wacker B, Bueren E, Ham E, Dronadula N, Dichek DA. A rabbit model for testing helper-dependent adenovirus-mediated gene therapy for vein graft atherosclerosis. Molecular Therapy: Methods and Clinical Development. 2017;7:96–111 [PubMed: 29296626]
11. Bi L, Wacker BK, Dichek DA. A rabbit model of durable transgene expression in jugular vein to common carotid artery interposition grafts. J Vis Exp. 2018
12. Pan D, Gunther R, Duan W, Wendell S, Kaemmerer W, Kafri T, Verma IM, Whitley CB. Biodistribution and toxicity studies of vsvg-pseudotyped lentiviral vector after intravenous administration in mice with the observation of in vivo transduction of bone marrow. Mol Ther. 2002;6:19–29 [PubMed: 12095299]
13. Park F, Ohashi K, Chiu W, Naldini L, Kay MA. Efficient lentiviral transduction of liver requires cell cycling *in vivo*. Nat. Genet. 2000;24:49–52 [PubMed: 10615126]
14. VandenDriessche T, Thorrez L, Naldini L, Follenzi A, Moons L, Berneman Z, Collen D, Chuah MK. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. Blood. 2002;100:813–822 [PubMed: 12130491]
15. Esteban V, Mendez-Barbero N, Jimenez-Borreguero LJ, Roque M, Novensa L, Garcia-Redondo AB, Salaices M, Vila L, Arbones ML, Campanero MR, Redondo JM. Regulator of calcineurin 1 mediates pathological vascular wall remodeling. J. Exp. Med. 2011;208:2125–2139 [PubMed: 21930771]
16. Oller J, Mendez-Barbero N, Ruiz EJ, et al.Nitric oxide mediates aortic disease in mice deficient in the metalloprotease adamts1 and in a mouse model of marfan syndrome. Nat. Med. 2017;23:200–212 [PubMed: 28067899]
17. de Yebenes VG, Briones AM, Martos-Folgado I, et al.Aging-associated mir-217 aggravates atherosclerosis and promotes cardiovascular dysfunction. Arterioscler. Thromb. Vasc. Biol. 2020;40:2408–2424 [PubMed: 32847388]
18. Huang X, Yue Z, Wu J, Chen J, Wang S, Wu J, Ren L, Zhang A, Deng P, Wang K, Wu C, Ding X, Ye P, Xia J. Microrna-21 knockout exacerbates angiotensin ii-induced thoracic aortic aneurysm and dissection in mice with abnormal transforming growth factor-beta-smad3 signaling. Arterioscler. Thromb. Vasc. Biol. 2018;38:1086–1101 [PubMed: 29519942]
19. Lovren F, Pan Y, Quan A, Singh KK, Shukla PC, Gupta N, Steer BM, Ingram AJ, Gupta M, Al-Omran M, Teoh H, Marsden PA, Verma S. Microrna-145 targeted therapy reduces atherosclerosis. Circulation. 2012;126:S81–90 [PubMed: 22965997]
20. Xu X, Zhang F, Lu Y, Yu S, Sun W, Sun S, Cheng J, Ma J, Zhang M, Zhang C, Zhang Y, Zhang K. Silencing of nono inhibits abdominal aortic aneurysm in apolipoprotein e-knockout mice via collagen deposition and inflammatory inhibition. J Cell Mol Med. 2019;23:7449–7461 [PubMed: 31512366]
21. DuPont JJ, McCurley A, Davel AP, McCarthy J, Bender SB, Hong K, Yang Y, Yoo JK, Aronovitz M, Baur WE, Christou DD, Hill MA, Jaffe IZ. Vascular mineralocorticoid receptor regulates microrna-155 to promote vasoconstriction and rising blood pressure with aging. JCI Insight. 2016;1:e88942 [PubMed: 27683672]
22. Wacker BK, Bi L, Dichek DA. In vivo gene transfer to the rabbit common carotid artery endothelium. J Vis Exp. 2018;135:56982
23. Sohlenius-Sternbeck AK. Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. Toxicol In Vitro. 2006;20:1582–1586 [PubMed: 16930941]
24. Mizen NA, Petermann ML. Nuclei from normal and leukemic mouse spleen. Iii. The desoxypentosenucleic acid content per nucleus calculated from total cell counts. Cancer Res. 1952;12:727–730 [PubMed: 12988201]
25. Colvin GA, Lambert JF, Abedi M, Hsieh CC, Carlson JE, Stewart FM, Quesenberry PJ. Murine marrow cellularity and the concept of stem cell competition: Geographic and quantitative determinants in stem cell biology. Leukemia. 2004;18:575–583 [PubMed: 14749701]

26. Follenzi A, Sabatino G, Lombardo A, Boccaccio C, Naldini L. Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. Hum. Gene Ther. 2002;13:243–260 [PubMed: 11812281]

27. Ohashi K, Park F, Kay MA. Role of hepatocyte direct hyperplasia in lentivirus-mediated liver transduction in vivo. Hum. Gene Ther. 2002;13:653–663 [PubMed: 11916488]

28. Tsui LV, Kelly M, Zayek N, Rojas V, Ho K, Ge Y, Moskalenko M, Mondesire J, Davis J, Roey MV, Dull T, McArthur JG. Production of human clotting factor ix without toxicity in mice after vascular delivery of a lentiviral vector. Nat. Biotechnol. 2002;20:53–57 [PubMed: 11753362]

29. Nosek BA, Errington TM. The best time to argue about what a replication means? Before you do it. Nature. 2020;583:518–520 [PubMed: 32694846]