NEW METHODS FOR AORTIC DISEASE MODELING USING LENTIVIRAL VECTORS

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Abstract

Lentiviral vectors (LV) transduce quiescent cells, and provide stable integration to maintain transgene expression. Several approaches have been adopted to optimize LV safety profiles. Similarly, LV targeting has been tailored through strategies including the modification of envelope components, the use of specific regulatory elements, and the selection of appropriate administration routes. Models of aortic disease based on a single injection of pleiotropic LVs have been developed which efficiently transduce the three aorta layers in wild type mice. This approach allows the dissection of pathways involved in aortic aneurysm formation and the identification of targets for gene therapy in aortic diseases. LVs provide a fast, efficient, and affordable alternative to genetically modified mice to study disease mechanisms and develop therapeutic tools.

Novel Applications of Lentiviral Vectors

Lentiviruses (LV) are a type of retrovirus able to transduce quiescent cells, although this process is less efficient than transduction of stimulated or low proliferative cells such as hematopietic stem cells (HSCs). These viruses express reverse transcriptase, which transforms viral RNA into DNA. Viral DNA stably inserts into the host genome through the action of viral integrase. Engineered LVs optimized to fulfill biosafety requirements, with improved target specificity and more efficient **transduction** (see Glossary), constitute effective tools for long-term gene modification *in vivo*. LVs avoid several major drawbacks associated with gene delivery through **adenovirus** (AdV) or **adeno-associated virus** (AAV) systems, such as the induction of an intense inflammatory response and transient transgene expression in the case of AdVs and the complex production protocols and low cargo capacity of AAVs (e.g., while LV packaging capacity is up to 8Kb, AAV cargoes cannot exceed 4.5Kb). LVs efficiently transduce nondividing differentiated cells and stably integrate into the host-cell genome while generating only a moderate immune response (Table 1)[1,2,3]. These features make LVs optimal tools for a number of purposes, including gene therapy and *in vivo* dissection of molecular pathways involved in pathophysiological processes. In this opinion article, we examine general topics related to the *in vivo* applications of LVs, with particular emphasis on safety concerns and methods to improve target selectivity. We propose applications of LVs in the identification of therapeutic targets and the evaluation of candidate treatments, as well as the potential use of LV transduction for gene therapy in aortic diseases. Finally, we discuss our view that these unique LV properties make these vectors an optimal system for disease model development.

Biosafety Issues

The occurrence of **leukemogenesis** due to **insertional mutagenesis** in either X-linked SCID or Wiskott-Aldrich syndrome patients treated with **gammaretroviral vectors** [4,5] reinforced the importance of biosafety as a central concern in gene therapy. Strategies aimed at achieving satisfactory biosafety levels in LV applications *in vivo* have been progressively adopted, leading to successful administration of LV for gene therapy of diseases as Wiskott-Aldrich syndrome, X-adrenoleukodystrophy, metachromatic leukodystrophy, and others [5,6,7]. This approach has given rise to successive generations of LVs with increasingly sophisticated biosafety characteristics. For example, modification of LV elements has helped to reduce critical risks, such as LV replication *in vivo* leading to undesired transduction events, and

insertional mutagenesis that could lead to cancer. Intercellular transmission of vector components beyond first-line transduction needs to be prevented in order to avoid the spread of potentially deleterious sequences. Approaches used to achieve this include the generation of a split viral genome, together with the deletion of accessory genes, aimed at reducing the production of replication-competent viral particles in packaging cells. Thus, LV genome is usually distributed into several plasmids, which comprise a transfer plasmid (encoding the transgene of interest), one or two packaging plasmids, and an envelope plasmid. In 3rd generation LV, most original viral packaging components are removed, and the remaining are distributed in two plasmids; besides, to overcome the elimination of Tat protein, a Tat-independent 5' LTR is included in the transfer plasmid. Additionally, strategies leading to *ad hoc* modification of LV tropism restrict transduction to specific cell types (see below), and contribute to limit off-target effects [8]. LVs usually integrate into transcriptionally active genomic areas, but unlike gammaretroviruses they show no preference for promoter regions. This integration pattern is associated with the activity of LEDGF/p75, which links host DNA to LV integrase [9,10]. However, insertional mutagenesis upon LV integration remains a critical safety concern, and different strategies have been adopted to reduce this potential risk, such as the elimination of enhancer/promoter sequences from the LTRs to produce self-inactivating (SIN) LVs. Similarly, **integration-deficient LVs** (IDLV) with class I integrase mutations and a low integration rate are being developed. IDLV remain as **episomes** in the host cell, and are thus suitable for sustained gene expression in quiescent cells or transient modification of proliferative cells [11]. Despite the safer profile of IDLV, several concerns have arisen related to their *in vivo* efficacy. Hence, transduction with IDLV yields lower transgene expression as compared to LV; also, long-term follow up studies have reported a substantial decrease in transgene expression in mice after 1 year in organs as liver and cerebellum, whereas in retina sustained GFP expression has been reported for at least 9 months [11,12,13]. Combinations of IDLVs encoding Zn finger nucleases and a DNA template of interest have been used successfully for safe site-directed integration of exogenous DNA sequences into the host genome [14]. Further approaches to circumvent insertional mutagenesis include the generation of **self-deleting LVs** and transposon hybrids [15] (Box 1).

A major restraint for **systemic LV administration** *in vivo* is the activation of host innate and adaptive immune responses to LV components or the transgene product, which can lead to the clearance of LVs or LV-transduced cells, as shown in baboons subjected to myeloablative

conditioning [16]. Systemic inoculation with LVs triggers a transient type I IFN increase in mouse liver, mainly mediated by the recognition of viral ssRNA by TLRs, especially TLR3 and TLR7. This response, probably initiated by plasmacytoid dendritic cells, limits transduction of and transgene expression in hepatocytes *in vivo* [17,18]. Additionally, the induction by IFN of some **restriction factors,** like tetherin, effectively prevents virus replication in host cells, as demonstrated in HeLa cell line [19]. Specific immune responses against exogenous transgene-encoded proteins also interfere with *in vivo* LV efficiency; however, this issue can be avoided by introducing target sequences for dendritic cell-specific miRNAs into the 3' UTR of the transgene of interest. The suppression of transgene expression through this approach largely depends on a threshold amount of endogenous miRNA, which can be exploited to selectively target cells in a particular functional state [20]. Additionally, specific targeting of LVs to hepatocytes may circumvent the host immune response. This strategy has been successfully used to achieve sustained production of human factor IX in a murine model [21]. Interestingly, mass spectometry analysis of MHC-I in human HEK 293T, D407, and HER 911 cell lines transduced with LVs *in vitro* reveals a lack of MHC-I-presented transgene-derived peptides, together with changes in the repertoire of MHC-I-presented self-peptides. This response could promote an autoimmune condition in susceptible individuals [22].

In summary, although engineering of viral components reduces biosafety risks and the triggering of host immune responses, the development of potential LV-related side effects is an eventuality that must be taken into consideration. Long-term monitoring of patients undergoing LV-based gene therapy will be critical to assess the actual incidence and nature of these events, and to advance new strategies to optimize LV characteristics.

Targeting strategies

A priority in disease modeling with LVs is to develop strategies to ensure spatial or temporal restriction of vector delivery and transgene expression, thus avoiding transfer of genetic material to off-target or potentially dangerous cells. Below, we discuss major **targeting** strategies focused either on engineering LV components to efficiently re-direct LV particles toward the desired cell type or on the selection of specific LV administration routes.

Main targeting strategies

LV particles can be selectively targeted either by modifying vector surface characteristics to

optimize LV binding to the target cell or by including regulatory sequences in the cargo whose expression is restricted to the selected target cell type. **Viral tropism** is primarily dependent on glycoproteins present in the LV envelope, since these are recognized by membrane receptors not expressed on all cells. For instance, the VSV-G envelope binds to the LDL receptor; since this receptor is absent from the surface of resting T lymphocytes, these cells are resistant to VSV-G transduction [23]. LVs can thus be engineered to express particular envelope glycoproteins (pseudotyped) to either induce or avoid preferential binding to selected cell types. VSV-G is the most frequently used virus for pseudotyping, although other viral envelopes are also used, such as rabies, measles virus, Ebola, and Sindbis virus [24]. However, precise LV targeting usually involves the generation of chimeric glycoproteins fused to ligands, IgG, or single-chain variable fragments (scFvs), which recognize specific cell surface receptors. Envelope glycoproteins with separate membrane fusion and receptor binding functions are preferentially used for this approach, as in the case of Sindbis and measles virus [25,26].

Targeting of vascular cells

Vascular disease can affect different vascular beds throughout the body and includes a variety of entities, including atherosclerotic peripheral and carotid arterial diseases, aortic aneurysm, Raynaud's phenomenon, and Buerger disease. LV transduction of vascular cells *in vivo* allows the investigation and manipulation of specific molecular mechanisms involved in vascular disease progression and the identification of potential therapeutic targets. LV-based approaches have been used either to prevent disease or to develop preclinical models of diseases that occur with pathological vascular wall remodeling, such as atherosclerosis, restenosis, and aortic aneurysm. These approaches have led to the identification of disease-mediating pathways, particularly for aortic aneurysm [27,28,29].

Most blood vessels are composed of three layers: the tunica intima (inner layer) consists of a single layer of ECs supported by a subendothelial connective tissue layer; the tunica media (middle layer) contains circular elastic fibers, connective tissue, and VSMCs; the tunica adventitia, the outermost layer, is formed of connective tissue. Capillaries lack the media and adventitia, having only a single layer of ECs and connective tissue.

Within blood vessels, ECs cover the entire luminal vessel surface, and are therefore potentially more accessible to transduction by parenteral LV administration without specific targeting. Accordingly, functional data demonstrate efficient transduction and gene

modification of mouse aorta ECs upon systemic injection of **pleiotropic LVs** through the jugular vein. LVs transduce all three aortic layers efficiently, and histology at very early post-inoculation stages -1 day after transduction- reveals transduction of ECs, VSMCs, and cells of the adventitia (Fig. 1, 2). In our opinion, these results indicate that transduction does not spread gradually from the intima to the adventitia, but rather that LVs can transduce cells of the outer layers of the vascular wall through additional routes that do not necessarily involve intima transduction, such as potential LV access to adventitia through *vasa vasorum* [27,28,30]. In addition, ECs from different vascular beds display heterogeneous functional characteristics *in vivo* [31], which can be exploited to selectively target a specific blood vessel type for analysis. For example, LVs engineered to express an scFv specific for the EC marker endoglin (CD105) show high selectivity for liver sinusoidal ECs after intravenous (i.v.) tail-vein inoculation in mice [32]. The incorporation of EC-specific regulatory elements into the LV-encoded cargo sequence has also achieved efficient specific EC targeting *in vivo*. Several EC-specific promoters were tested, including those from the Tie1, Tie2, Flk-1, VE-Cadherin, and ICAM-2 genes; of these, the Tie2 promoter/enhancer -also active in HSCs and monocytes- was the most EC-selective, driving preferential transgene expression in ECs *in vitro* and in the small tumor vasculature after systemic tail-vein LV injection in mice [33]. Similarly, specific modification of TLR4 expression in lung ECs has been achieved with the VE-cadherin promoter [34]. Interestingly, combination of several targeting strategies significantly enhances specificity. Thus, dual-targeted LVs carrying a surface anti-CD146 antibody and simultaneously encoding the Tie2 promoter/enhancer showed higher specificity than single-modified LV versions in primary human ECs *in vitro* [35].

VSMCs have been successfully targeted using LVs pseudotyped with the Hantavirus envelope in a rabbit model of carotid injury [36] and through the cloning of the SMC-specific promoter SM22α, which specifically drives expression of miRNA-145 in aortic VSMCs after tail-vein LV injection in *Apoe-/-* mice [37]. In addition, intratracheally instilled LVs bearing a miRNA targeting the SM22α gene—a cytoskeleton protein involved in vascular remodeling and hypoxic pulmonary hypertension—specifically modulate gene expression in pulmonary arterial SMCs. In this case, LV specificity resides in selective expression of SM22α in this cell type [38]. Many authors suggest that *in vivo* VSMC transduction largely depends on the loss of EC layer integrity, for example by mechanical disruption with a balloon catheter introduced into the carotid artery and inflated *in situ* to produce EC injury [36]. Indeed, ECs are considered a barrier

to LVs that is only overcome by EC injury or in specialized areas such as capillary beds, liver sinusoids, or kidney glomeruli. LV entry is impeded by adherens junction formation in epithelial cells, and this effect is blocked by E-cadherin knockdown. VE-cadherin has been proposed to play a similar role in virus traffic through the EC layer [39,40]. Interestingly, intrajugular inoculation of VSV-G-pseudotyped LVs in several mouse models (wild type, *Apoe* -/-, and MT4-MMP deficient mice) has yielded functional VSMC transgene expression of GFP-fused calcineurin-blocking LxVP peptide, Adamts-1 shRNA, and MT4-MMP. This LV-based approach has allowed to dissect the role of VSMCs in aortic disease development (Fig.1, 2) [27,28,29]. VSMC transduction *in vivo* has also been reported upon tail-vein injection of LV encoding Smad7 shRNA or miR-29b pre-miR constructs [41,42]. The access route to VSMC has not been delineated, but several mechanisms could account for the efficient targeting of these cells. For example, in experiments conducted in *Apoe* -/- or *Smad3*+/- mice, EC dysfunction may have altered barrier function to facilitate LV transduction of the medial layer (Fig. 2) [27,41,42]. Furthermore, VSMCs have been successfully transduced by intrajugular LV inoculation in MT4-MMP-deficient neonatal mice, which may allow easier LV access through the EC barrier (Fig.2) [29]. However, a similar degree of VSMC transduction can be observed in wild type adult C57BL/6 mice when knocking down Adamts1 to address the role of this metalloproteinase in aortic aneurysm and medial degeneration (Fig.1, 2). Thus, VSMC transduction has been achieved empirically by intrajugular injection of pleiotropic LVs. In our opinion these data refute the hypothesis that viral vectors can transduce VSMC only if endothelial permeability is altered, a hypothesis based on data obtained with AdV or LV in preclinical models of carotid injury, which show VSMC transduction only in areas of EC denudation [34,43]. We consider that the lack of VSMC transduction upon tail-vein injection reported by some groups could be due to differences in the administration route, viral vector type, and virus titer. For instance, viruses have been inoculated systemically through the tail vein [44,45,46] or locally into the target vessel [34,47,48], whereas in the models discussed above LVs are delivered by intrajugular injection [27,28,39], which could affect viral vector distribution. Many groups have used AdVs instead of LVs to modulate *in vivo* gene expression [43,45,47,48], and VSMC transduction efficiency may also be influenced by differences in viral vector tropism or biodistribution. Furthermore, inoculation of different amounts of viral particles could partially account for discrepancies in *in vivo* distribution. Studies analyzing the number of AdV particles required to efficiently transduce ECs *in vivo* have determined an optimal dose of 10^{10}-10^{11}

infective particles per animal to achieve a maximal transgene expression in target cells [47]. In contrast, the number of infective LV particles employed in most *in vivo* analyses is around 10^7 per animal [37,44,49], although the relative transduction efficiency between AdVs and LVs has not been firmly established. In studies mentioned above, successful transduction of VSMCs has been achieved by inoculating 10^8 active LV particles per mouse, which could explain the efficient transduction of all aortic layers [27,28,29]. Additional processes may have allowed VSMC transduction in these assays. For example, VSV-G-pseudotyped particles can be retained in fibronectin-rich extracellular matrix and transmitted to secondary cellular targets [50]. Moreover, viral particle **transcytosis** has been described for adenovirus (e.g. transferrin receptor-mediated transcytosis in Caco-2 cells), HIV, and HTLV1 in an *in vitro* model of epithelial tight barrier [51,52]. Hence, despite the actual mechanisms involved have not been determined yet, our view is that VSMCs are clearly susceptible of transduction by LV in the presence of a conserved endothelial layer; and that the study of the mechanisms responsible for this will provide invaluable information on LV behavior *in vivo*. Several LV targeting strategies have been used to transduce other cell types, including dendritic cells [53], lymphocytes [54], astrocytes [55], tumor cells [56], and hematopoietic progenitors [57].

Relevance of the administration route

LVs can be delivered either systemically—usually by intraperitoneal or intravenous injection—or locally to specific tissues or organs where the gene modification is desired. The choice of a particular administration route also contributes to the organ and tissue distribution of LV particles. As mentioned, systemic inoculation usually requires modification of viral tropism or the incorporation of specific regulatory sequences to ensure selective transduction of target cells. However, in some cases, systemic administration of LVs through a specific route is enough to favor tropism toward a defined cell type. For example, in a mouse model of inflammatory arthritis, intraperitoneal injection preferentially targets pleiotropic VSV- G-pseudotyped LVs to activated macrophages [58]. This might be due to the intrinsic tropism of LVs for myeloid-lineage cells [59], but could also reflect the activation state of macrophages in these experiments, which might make them more susceptible to LV transduction. Furthermore, local administration protocols have been developed to limit spatial LV distribution. Locally restrained LV delivery generally leads to transduction of several cell types, which may provide a route to study the coordinated contribution of these cells to a given process or the global

effect of a genetic modification on the selected organ or structure. Local administration can also be combined with LV engineering to analyze the role of a specific cell type in the selected location. Examples of local LV delivery include intramyocardial administration in adult rats [60], intrafemoral injection for *in situ* transduction of hematopioetic stem cells in mice [61], intrathymic inoculation to target T-cell precursors in ZAP-70–deficient mice [62], and stereotactic injection at specific brain areas in rodents [63].

Lentiviral vectors in aortic disease modeling

The *in vivo* applications of LVs depend on their capacity for stable delivery, whether the cargo is single genes under ubiquitous or lineage-restricted regulatory elements, complex genetic structures in polycistronic cassetes, or shRNAs for functional interrogation of genes of interest. These characteristics, combined with the easy modulation of their immunogenicity and biodistribution properties, make them an invaluable tool in basic and translational research. The wide-ranging applications of LVs include LV-mediated transgenesis, immune modulation and vaccine development, *in vivo* cell imaging and lineage tracking, and reprogramming of pluripotent cells (reviewed in 2). Furthermore, LVs are now emerging as a useful means for dissecting molecular pathways involved in pathophysiological conditions and the design of preclinical models of disease. Conventional validation of putative targets based on genetic ablation of endogenous genes has provided invaluable information on disease mechanisms; however, our notion is that this approach is expensive and has limited versatility, since it is hard to achieve accurate temporal regulation of gene depletion in one or more cell types located in a given tissue or organ. We thus believe that there is a clear need for affordable and versatile disease models that support precise, spatio-temporally regulated gene modification *in vivo*.

LVs and preclinical models of aortic disease

Mouse models of **aortic aneurysm** have advanced from giving a merely mechanistic perspective—for example, through chemically-induced abdominal aortic aneurysm with elastase or calcium chloride infusion [64,65]—to providing information on molecular pathways involved in aortic aneurysm progression in knockout mice. Mice deficient in Lox, MMP-3, TIMP-1, LDL receptor, or ApoE develop abdominal aortic aneurysm in basal conditions or upon Ang II treatment [66-69]. Similarly, mouse models of **Marfan syndrome** (MFS), which carry a knock-

in mutation in the *Fbn1* locus, develop thoracic aortic aneurysm associated with altered TGFβ and AngII pathways [70]. However, these models do not allow gene ablation at a specific stage of aortic aneurysm progression, which could help to define the specific stages when mediators act. The generation of inducible, tissue-specific knockout mice is a tedious, time-consuming and often leaky approach. By silencing *Adamts1* through LV-mediated shRNA delivery at a defined time point, characteristic changes in medial architecture may be induced together with strong dilation of the aortic ring, ascending aorta, and abdominal aorta in wild type mice, with progression to aneurysms in 13 of 16 animals and lethal dissections in 4 of 16 mice upon Ang II treatment [28]. This LV-based strategy allowed to determine the pathogenic sequence of thoracic aortic aneurysm generation, with Adamts1 participating in blood pressure changes and matrix degradation at early stages followed by involvement of the TGF-β–Smad pathway. This fast and highly controlled approach also led to the identification of nitric oxide (NO) as an essential thoracic aortic aneurysm mediator [28]. Using a similar approach, MT4-MMP activity has been shown to regulate the VSMC contractile phenotype and to be essential for preventing thoracic aortic aneurysm development [29]. Moreover, systemic intrajugular inoculation of pleiotropic LVs allowed identifying the decisive role of VSMCs in thoracic aortic aneurysm, despite the putative barrier function of ECs. While this approach cannot achieve specific cell targeting, further LV modification could enable dissection of the contribution of specific cell types at selected stages. The adequate transgene expression window for the accurate modeling of aortic disease may be further determined with LV bearing drug-inducible promoters, as those regulated by doxycycline-dependent transactivators in Tet On/Tet off systems [71]. This method allows switching on and off transgene expression at desired times, and analyzing the pathogenic role of a given molecule at a specific point of disease development. Tet–based systems can be combined with tissue-specific promoters driving transactivator expression [72], which facilitate the analysis of temporal involvement of selected cell types in disease onset and evolution. With knockout technology, this would be barely achievable and would require the generation of mouse strains with multiple conditionally inducible floxed alleles, which is definitely a time-consuming, expensive and technically challenging approach.

LVs as therapeutic tools for aortic disease

In addition to disease modeling, we postulate that this strategy can be advantageously used

for therapy. LV integration leads to sustained transgene expression, and therefore a single inoculation of these vectors could achieve stable gene modification in chronic diseases. Indeed, transgene expression in the aortic wall has been detected for at least 49 days after intra jugular LV inoculation [28]. Although a longer and more sustained transgene expression can be anticipated, new cells might be recruited to inflamed or remodeling tissues at later stages, after LV administration. In this scenario, repeated LV doses may be needed to achieve a prolonged beneficial effect *in vivo*. On the other hand, the use of inducible LV systems (see above) may be very useful to establish the appropriate therapeutic window, and to provide a way to rapidly switch off the expression of the transgene in case a severe side effect is detected. Cardiovascular diseases are currently being targeted in several gene therapy clinical trials (e.g. NCT03039751, NCT02844283, NCT01002430, NCT00787059, NCT00620217, NCT00117650, and NCT00000431), but none of them addresses the treatment of aortic disease (www.clinicaltrials.gov). Data from genetically modified preclinical models suggest that specific gene targeting through viral vectors could be an effective therapeutic approach for aortic aneurysm [73]. Supporting this view, LV-mediated re-expression of MT4-MMP in VSMCs is enough to prevent aneurysm formation in MT4-MMP–deficient mice [29]. Likewise, an LV-based approach has been used to express peptide sequences that specifically block abdominal aortic aneurysm development and inflammation [27,58,74]. We believe that this experimental evidence provides a valuable *in vivo* proof-of-concept that encourages the use of LVs for gene therapy in human aortic disease. For example, recent findings show that pharmacological NOS2 inhibition results in LV-mediated regression of aortic dilation in MFS mice [28]. Given the preferential transduction of aorta by intrajugular-delivered LVs, NOS2 silencing may represent a gene therapy alternative that would circumvent potential side effects derived from systemic treatment with NOS2 inhibitors.

LV-based gene therapy is an emerging field with promising results in the treatment of a number of human diseases. However, LV application in the therapy of aortopathies still needs to overcome some obstacles. Most of LV-based therapies are currently carried out through *ex vivo* transduction of target cells (e.g. HSCs, T lymphocytes), which are subsequently inoculated back in patients. This procedure leads to a lower risk of systemic secondary effects derived from off-target transduction events, and requires a reduced amount of LV particles. Although direct *in vivo* inoculation of LV is also being conducted to treat some conditions as retinopathy and Parkinson's disease [75,76], therapy of those aortic diseases with VSMC involvement

probably requires large amounts of GMP-produced LV, and needs a careful LV design to limit the generation of side effects derived from systemic LV administration (see above). In addition, unlike monogenic diseases as β-thalassemia or Wiskott Aldrich syndrome, a precise dissection of mediators involved in aortic disease and their functional networks is needed prior to the application of LV in gene therapy of aortopathies. As previously discussed, LV are optimal tools for the establishment of aortic models of disease and for a thorough analysis of mediators involved in their pathogeny, and constitute a reliable system for preclinical assessment of gene therapy suitability.

Concluding remarks

LVs have unique features that make them suitable for safe, long-term *in vivo* gene modification and can be easily engineered to improve biosafety and refine cell tropism. LVs are also emerging as valuable tools for dissecting molecular pathways involved in disease and can be applied to the design of efficient preclinical disease models. LV-based approaches have significant advantages over conventional gene ablation strategies. Unlike knockout models, LVs provide fast, time-controlled, and cell-, tissue- or even region-restricted gene expression modulation and allow simultaneous evaluation of the impact of gene manipulation in selected cell types. These characteristics show the singular properties of LVs not only for disease modeling, but also for evaluating the effectiveness of therapeutic gene modification and candidate molecules in preliminary stages of drug development (see Outstanding Questions).

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ABBREVIATIONS

AAV: Adeno associated virus

AdV: Adenovirus

Ang II: Angiotensin II

ApoE: Apolipoprotein E

EC: Endothelial cell

GMP: Good Manufacturing Practices

HIV: Human immuno deficiency virus

HTLV1: Human T lymphotrophic virus or human T-cell leukemia-lymphoma virus.

HSC: Hematopoietic stem cell.

IDLV: Integration-deficient lentiviral vector

IFN: Interferon

LDL: Low density lipoprotein

LEDGF: Lens epithelium-derived growth factor

Lox: Lysyl oxidase

LTR: Long terminal repeat

LV: Lentiviral vector

MHC: Major histocompatibility complex

MMP3: Matrix metalloprotease 3

MT4-MMP: Membrane type-4 matrix metalloproteinase

NOS2: Nitric Oxide Synthase 2

SCID: Severe combined immunodeficiency

ssRNA: Single-stranded RNA

TGF: Transforming growth factor

TIMP: Tissue inhibitor of metalloproteinase

TLR: Toll like receptor

LTR: Long terminal repeat

VSMC: Vascular smooth muscle cell

VSV-G: glycoprotein G of the vesicular stomatitis virus

ZAP 70: Zeta Chain Of T Cell Receptor Associated Protein Kinase 70

GLOSSARY

Adenovirus: Non-integrative, double-stranded DNA viruses without an envelope, which transduce dividing or non-dividing cells and can cause respiratory disease in humans. Modified versions of these viruses are used as vectors for gene delivery.

Adeno-associated virus: Virus originally discovered as contaminants of adenovirus preparations. These single-stranded DNA viruses can infect both dividing and non-dividing cells and do not cause human disease.

Aortic aneurysm: Pathological dilation of the aorta secondary to an alteration in aortic wall components. Aneurysms can appear at several locations (aortic root, thoracic aorta, abdominal aorta). Aneurysm rupture has a high mortality rate.

Episome: Circular extra-chromosomal genetic material.

Gammaretroviral vectors: Gamma retroviruses are RNA viruses that express reverse transcriptase and integrase. The reverse transcriptase converts the viral RNA into DNA, and the integrase integrates this DNA integrates into the host cell genome. Gamma retroviruses infect only dividing cells.

Insertional mutagenesis: Generation of DNA mutations through the introduction of additional base pairs in the DNA sequence (in the case of LVs, mutations occur through the insertion of viral sequences into the host genome).

Integration-deficient LVs (IDLV): LVs unable to insert their DNA into the host genome. IDLVs have an inactive integrase (the enzyme responsible for integration of viral genetic material into the DNA of the transduced cell). IDLVs give rise to episomes, which are progressively lost with cell divisions, resulting in transient transgene expression.

Leukemogenesis: Process leading to the development of leukemia. Leukemogenesis is thought to be a multistep process involving structural and functional changes in a series of genes that result in the expansion of malignant cells.

Marfan syndrome (MFS): Genetic disorder that affects connective tissue, mainly due to mutations in the Fibrillin1 gene. The resulting connective tissue alterations lead to a

characteristic appearance of MFS patients, who are usually tall, thin, and have long limbs and flexible joints. Patients also frequently have scoliosis and an increased incidence of mitral-valve prolapse and aortic aneurysm, which is the major cause of death in these patients.

Pleiotropic LV: LVs with unrestricted tropism, which can transduce a variety of cell types.

Self-deleting LV: LVs that, after integration into the host-cell DNA, are flanked by LoxP sites recognized by the recombinase Cre. Cre activity leads to recombination and subsequent excision of the viral sequences from host DNA.

Systemic administration: Administration routes used to obtain a general distribution in the body. The route can be enteral (through digestive system) or parenteral (e.g. through intravenous or intraperitoneal injection).

Restriction factors: Cellular proteins that block key steps in the virus life cycle, such as viral replication.

Targeting: Modification of viral vector characteristics to achieve preferential transduction or transgene expression in a selected cell type.

Transcytosis: Transport of macromolecules and viral particles across a cell. Transcytosis is a frequent mechanism in ECs, in which the protein albumin is transported through a specialized transcytosis mechanism.

Transduction: Transfer of exogenous DNA into a cell by a viral vector.

Viral tropism: Selectivity of a virus for a given host tissue, partially dependent on the interaction of viral surface structures with host-cell-surface receptors.

BOX 1. HYBRID STRATEGIES FOR IMPROVING LV BIOSAFETY

Mutational inactivation of LV class I integrase—the enzyme required for stable integration of viral sequences into host-cell DNA—has allowed the generation of IDLVs. Unlike class I integrase mutants, mutants of class II integrase display additional defects in viral assembly and reverse transcription. IDLVs remain as episomes in host-cell nuclei, where they support efficient transgene transcription. IDLVs have been used to develop hybrid strategies aimed at reducing insertional mutagenesis by LVs.

1. Hybrid ZFN-LV techniques. Zinc-finger nucleases excise specific genomic DNA sequences by introducing double-strand breaks in the desired target gene. ZFNs have been used for targeted gene editing through homologous recombination. Simultaneous transduction of IDLVs encoding a ZFN and the DNA of interest allows the insertion of a particular transgene into a safe (e.g. noncoding) region of host DNA, thus minimizing the risk of random integration [77].

2. Hybrid transposon-LV approaches. The Sleeping Beauty (SB) transposon allows integration of a sequence of interest into the target-cell genome with no preference for transcriptionally active regions. This system involves the interaction between the SB transposon inverted repeats (with reduced promoter/enhancer activity) and SB transposase. The cotransduction of IDLVs encoding SB transposase and an expression cassette flanked by SB inverted repeats redirects transgene integration away from transcriptionally active regions, reducing the probability of insertional mutagenesis [78].

CLINICIAN'S CORNER BOX

- Modified lentiviruses are commonly used as vectors for *in vivo* gene manipulation in preclinical models because they integrate into the host cell DNA, thus providing stable gene modification. In addition, modified lentiviruses have a good biosafety profile and do not trigger an intense host immune reaction. These characteristics can be further improved by engineering particular viral components.
- Tropism of lentiviral vectors (LV) can be modified to selectively direct transgene expression toward a particular cell type. However, some groups have reported that effective transduction of VSMCs upon i.v. LV injection require additional EC injury. Nevertheless, efficient VSMC transduction has been achieved with intrajugular injection of LVs in the presence of an intact endothelium.

- LV-based systems have allowed to develop preclinical models of aortic aneurysm in normal mice in a rapid, versatile, and affordable manner. This approach also provides information about molecular mechanisms of aortic disease and potential therapeutic targets.
- LVs are being evaluated in clinical trials either alone or as vectors for therapeutic cell transduction. A recent example of therapeutic cell transduction is CAR-T cells, lymphocytes with an LV-modified chimeric antigen receptor. These have been successfully used to treat blood malignancies such as acute lymphoblastic leukemia in children and diffuse large B-cell lymphoma in adults. Studies discussed here provide further evidence for the potential of LV as therapeutic tools in aortic disease.

DISCLAIMER STATEMENT

The authors declare they have no competing interests.

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FIGURE LEGENDS

Figure1. Lentiviral-driven specific transgene expression in aortic layers. Mice received intrajugular injections of lentiviral vectors encoding either control or Adamts1 shRNAs and GFP protein. shRNAs expression was directed by hU6 promoter, and GFP by ubiquitous CMV promoter. Vectors were pseudotyped with VSVg envelope and 10^8 infective viral particles in 100μl were inoculated per mice. The pictures show staining with anti-GFP and anti-Adamts1 antibodies of aortic sections at several levels 1 day after inoculation, as indicated. Transgene expression can be observed in all layers along the aorta. Figures reproduced with permission from Springer Nature.

Figure2. Modulation of aortic aneurysm development through lentivirus-mediated genetic manipulation. **A.** Mice received intrajugular (i.j.) injections with lentiviral vectors

encoding either control or Adamts1 shRNAs, and were treated as indicated with vehicle (control) or angiotensin II (Ang-II). Graphs show the increase in maximum diameter of the aortic ring (*left*) and ascending aorta (*middle*) in mice injected with lentivirus encoding Adamts1 shRNA. *Right*, Staining of the ascending aorta with Masson's trichrome (Masson) (top), elastic van Gieson (EVG) (middle), and Alcian blue (bottom), revealing characteristic features of medial degeneration similar to those induced by Ang-II observed in Adamts1 shRNA-treated mice. Yellow arrowheads indicate elastin breaks. **B.** ApoE-/-mice were inoculated i.j. with lentiviral vectors encoding a GFP-fused form of the calcineurin blocking peptide LxVP or its mutant version LxVPmut. Mice were Ang-II treated to induce abdominal aortic aneurysm formation. *Left*, GFP expression in abdominal aortic layers. *Right*, Expression of LxVP, but not LxVPmut, significantly reduces the Ang-II-induced increase in abdominal aorta diameter. **C.** 1-day-old *Mmp17*-/- mice were inoculated i.j. with lentiviral vectors encoding WT or the E248A catalytically inactive version of Mmp17. Photographs correspond to transmission electron microscopy images of thoracic aortic sections, showing recovery of extracellular matrix structure in WT but not E248A mutant Mmp17-injected mice. Figures reproduced with permission from Springer Nature (**A**), Rockefeller University Press (**B**), and Wolters Kluwer (**C**).

Virus	Genome	Cargo Capacity	Cells Infected	Genome Integration	Expression	Immune response	Usual titer (particles/ml)	Main drawbacks
Adenovirus	Single stranded DNA	>8Kb	Dividing & Non dividing	No	Transient	Strong	10^{13}	Strong immune response
Adeno-associated virus	Double stranded DNA	≤4,5Kb	Dividing & Non dividing	0.1% frequency	Stable	Very low	10^{11}	Complex production protocols
Gamma retrovirus	RNA	<8Kb	Dividing	Yes	Stable	Low	10^{9}	Possible insertional mutagenesis
Lentivirus	RNA	<8Kb	Dividing & Non dividing	Yes	Stable	Low	10^{9}	Possible insertional mutagenesis

Table 1. Main characteristics of commonly used viral vectors

OUTSTANDING QUESTIONS

- What is the mechanism of VSMC targeting by LVs *in vivo*, in the presence of an intact EC layer?
- Is it possible that LVs cross the EC barrier by transcytosis?
- Can a specific administration route (eg, tail vein vs. intrajugular) modulate LV tropism and promote transduction of a particular cell type?
- Can LVs be used to model diseases other than aortopathies?
- Do LVs show the same tropism in patients as observed in preclinical models?
- Could aortic disease be reverted by gene therapy with a restricted aortic LV-transduction pattern?
- Are results obtained for LV delivery of therapeutic molecules in preclinical models applicable in a clinical setting?

Figure 1

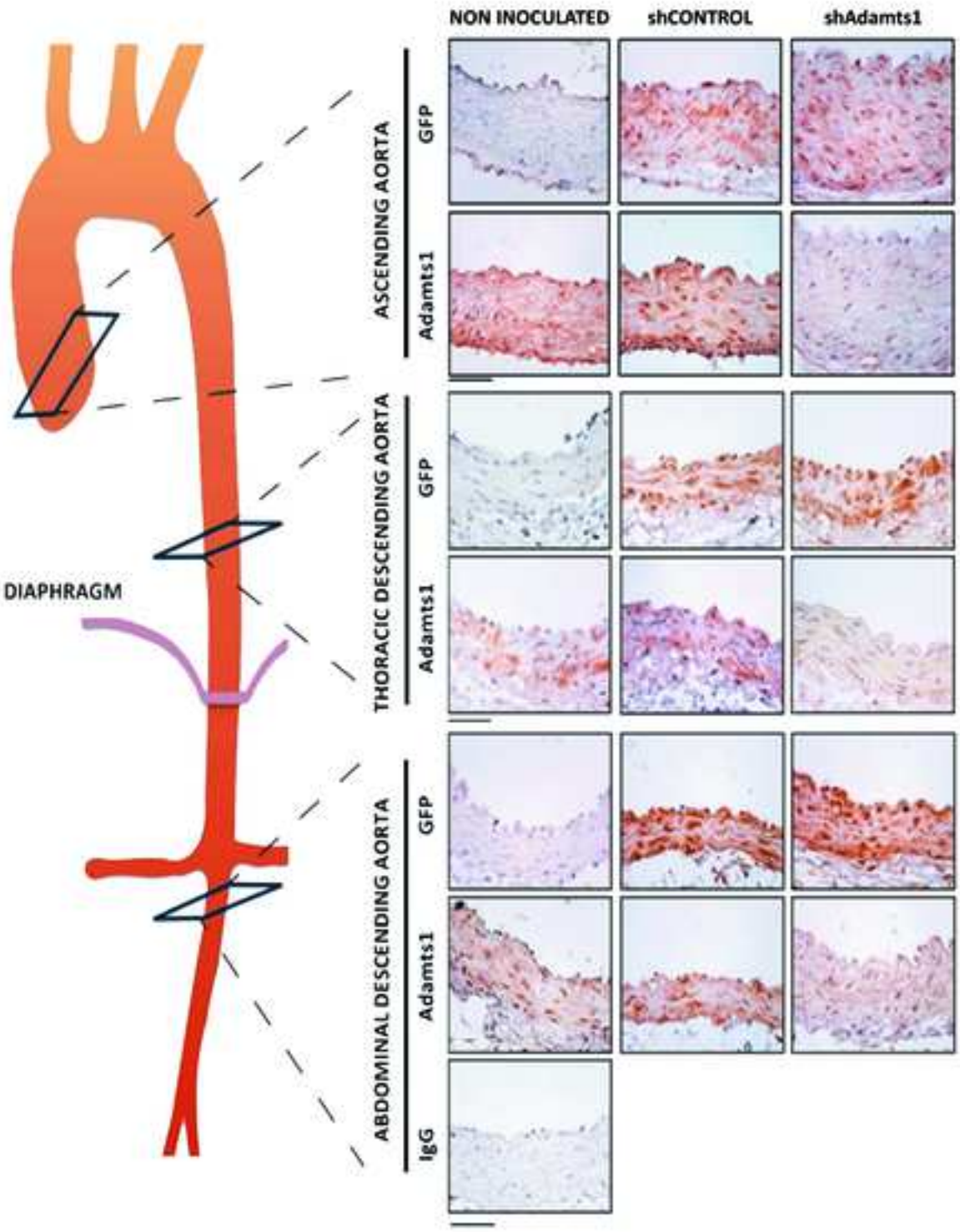
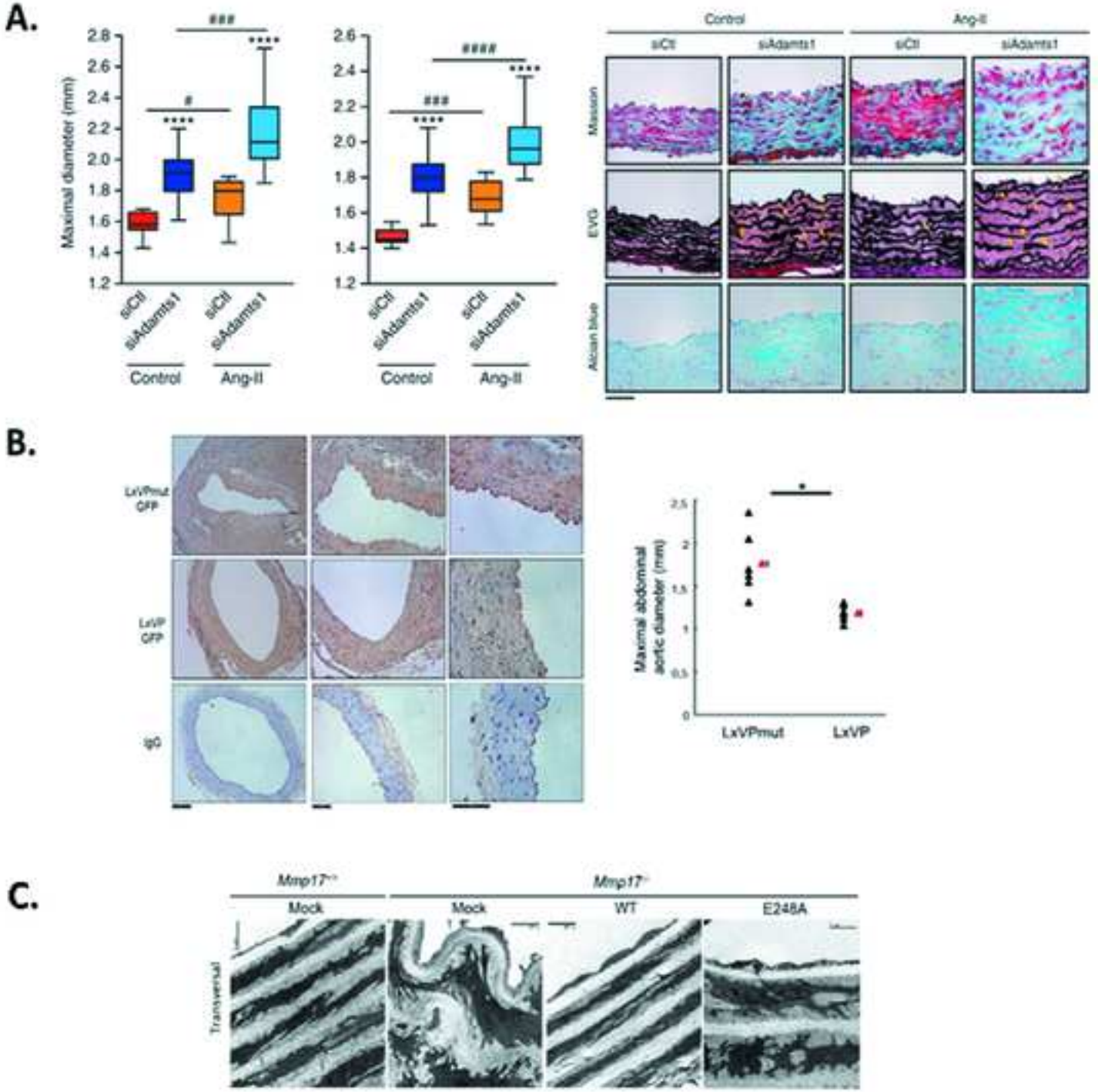

NON INOCULATED
shCONTROL
shAdamts1
ASCENDING AORTA
GFP
Adamts1
THORACIC DESCENDING AORTA
GFP
Adamts1
DIAPHRAGM
ABDOMINAL DESCENDING AORTA
GFP
Adamts1
IgG

Figure 2


A.
Maximal diameter (mm)
siCtl
siAdamts1
Control
Ang-II
Control
Ang-II
Masson
EVG
Alcian blue
B.
LxVPmut GFP
LxVP GFP
IgG
Maximal abdominal aortic diameter (mm)
LxVPmut
LxVP
C.
Mmp17-/-
Mmp17-/-
Mock
Mock
WT
E248A
Transversal