Effective Gene Delivery to Valvular Interstitial Cells Using Adeno-Associated Virus Serotypes 2 and 3

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Currently, curative therapies for heart valve diseases do not exist, thus motivating the need for new therapeutics, regenerative and tissue-engineered valves, and further basic research into pathological mechanisms. For studying valve diseases and developing valve therapies, effective methods to manipulate gene expression in primary valvular interstitial cells (VICs), which promote calcification in disease, would be valuable. Unfortunately, there is little information reported about effective gene delivery methods for VICs. Adeno-associated virus (AAV) is a clinically proven gene delivery vector capable of transducing many cell types and tissues, but has not yet been reported to infect valvular cells. In this study, AAV serotypes 1–9 were tested for their ability to deliver a green fluorescent protein (*GFP*) reporter into VICs *in vitro*. Flow cytometry results indicate AAV2 and AAV3 are capable of transducing VICs more efficiently than other serotypes. Furthermore, transduction efficiencies can be optimized by increasing the multiplicity of infection (MOI) and using self-complementary, double-stranded genomes, yielding up to 98% successfully transduced cells. Transduction of VICs by AAV2 or AAV3 in the presence of competing soluble heparin significantly reduces delivery efficiencies, suggesting heparan sulfate proteoglycans act as the primary VIC receptors of these two serotypes. Overall, this study establishes AAV2 and AAV3 as efficient gene delivery vehicles for primary VICs. Such effective delivery vectors for valve cells may be broadly useful for numerous applications, including the study of valvular cell biology, development of valve disease therapies, and regulation of genes for tissue engineering heart valves.

Introduction

AORTIC VALVE DISEASE is the third most common cause of heart disease.¹ The prevalence is increasing and there is no curative therapy. As such, valve research has been pursued in multiple fields, including valvular biology and pathology, therapeutics for valve disease, and regenerative or tissue-engineered heart valves. A growing area of valvular research is the study of the most prevalent cell type in the valve, valvular interstitial cells (VICs). The primary function of VICs is to manufacture, remodel, and repair valvular extracellular matrix (ECM).² Investigators in the field of heart valve tissue engineering have studied VICs for their ability to assemble the complex architecture of valvular ECM. VICs also play a role in the pathogenesis of valve disease, wherein VICs display pathological phenotypes that demonstrate osteoblast-like behavior and elevated myofibroblast differentiation.³ This transition in cell phenotype is accompanied by a change in ECM in terms of composition, structure, and associated mechanical behavior, leading to altered and impaired valve function. The mechanisms of valve disease are not fully elucidated, and tissue

engineering approaches may be used to develop models of valve disease to study valve pathology in greater depth. The use of gene regulation in VICs also has the potential to be a valuable tool to regulate cell phenotype and direct ECM synthesis for tissue engineering, and to develop cellular therapies to treat disease.

Efficient, long-term gene delivery to VICs is a challenge, as it is with many types of primary cells. There are few studies demonstrating methods capable of highly efficient gene delivery to VICs, though many strategies have been reported. Lipid-based reagents that have been used for nucleic acid delivery to VICs include Lipofectamine 2000, a cationic lipid formulation to deliver DNA,⁴ siRNA,^{5–8} and miRNA mimics⁶; Dharmafect1, a cationic lipid-based vehicle for siRNA delivery⁹; and HiPerfect, a cationic lipid reagent, for siRNA delivery.^{10,11} Transfection reagents comprising lipids combined with other reagents have also been used, such as Oligofectamine, a lipid-polymer blend for siRNA delivery¹² and Turbofectin 8, a lipid-histone blend for DNA delivery. Nonliposomal vehicles have been reported, including nanoparticle-based N-TER for siRNA transfection¹³ and electroporation-based Nucleofector siRNA transfection system.^{14,15}

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Viral gene delivery vehicles used for VICs include lentivirus for shRNA interference,¹⁶ and adenovirus to deliver DNA.¹⁷ Although these methods were reportedly used successfully, these previous studies do not present comparable quantitative measurements of gene delivery efficiency, so it is difficult to assess which method is best for future studies.

Adeno-associated virus (AAV) is a clinically proven, nonpathogenic gene transfer vehicle capable of long-term gene expression. AAV-mediated gene therapy can persist up to 6 years in primate models.¹⁸ AAV has been shown to deliver genes to many cell types and tissues, including the heart, brain, and retina,¹⁹ but there have been no reports of using AAV for gene delivery to VICs. The goal of this study was to find an efficient gene delivery vehicle to VICs and provide a quantitative analysis of its transduction profile. In this work, a panel of AAV serotypes was screened for transduction in VICs and was further characterized in terms of dose dependence and single- versus double-stranded genomes.

Methods

Cell isolation

VICs were harvested from suckling pig aortic valves within 24 h postmortem from tissues obtained from a local commercial abattoir (Fisher Ham and Meats) as previously described.²⁰ Cells were harvested on two separate occasions from three to six aortic valves each harvest. The aortic valve leaflets were surgically removed and rinsed in phosphate-buffered saline (PBS) containing 5% antibiotic/antimycotic solution (ABAM; Cellgro). The leaflets were transferred into a collagenase-II solution (492 U/mL; Worthington) and placed in a shaking incubator for 30 min at 37°C to catalyze endothelial cell detachment. The surfaces of the leaflets were gently swabbed to remove the endothelial layer, and then leaflets were minced using surgical blades. The minced tissue was transferred to a collagenase-III solution (298 U/mL; Worthington) and incubated for 4 h at 37°C. The lower protease activity of collagenase-III minimizes cell damage while providing complete disaggregation.²⁰ Cells were filtered through a 40 µm strainer, pelleted by centrifugation, and resuspended in growth media (Dulbecco's modified Eagle's medium:F12, 10% bovine growth serum, 1% ABAM, and 1% 1 M HEPES) and seeded in tissue culture flasks. Growth media was replaced every 2–3 days. VICs used for experiments were limited to two to five passages.

Virus production

AAVs were produced as described previously.²¹ AAV1–AAV9 containing a *GFP* transgene driven by cytomegalovirus (CMV) promoter were produced by polyethylenimine (PEI)-mediated triple plasmid transfection of a *GFP* transgene (single-stranded: pITR-GFP, double-stranded: scAAV-CMV-GFP), helper plasmid (pXX6-80), and AAV *cap* gene plasmid (pXR1, pXX2, pXR3, pXR4, pXR5, pXR6, pXR7, pXR8, and pXR9) into HEK293T cells cultured in 15 cm dishes precoated with 0.001% poly-L-lysine (Sigma). Viruses were harvested and purified via iodixanol step gradient and ultracentrifugation as described previously.²¹ Helper-dependent serotype 5 adenoviral vectors (HDAd) were generously provided by Dr. M. Suzuki (Baylor College of Medicine, Houston, Texas).

Quantification of genomic titers

Virus genomic titers were measured with quantitative polymerase chain reaction (qPCR) involving SYBR Green dye and primers against the CMV promoter, as described previously.²¹ Three independent experiments were performed in duplicate.

Viral transduction assay

VICs were seeded on 24-well plates at a seeding density of 80,000 cells per well. After 24 h, viral vectors were added to the cells in serum-free media. (A pilot study showed no discernable difference between serum-free and serum-containing media during this incubation step. Data not shown.) Media was replaced with complete serum-containing medium 16 h post-transduction. Forty-eight hours post-transduction, cells were harvested by trypsinization for flow cytometry using FACSCanto II (BD Biosciences) to measure GFP-expression. Flow cytometry data were analyzed using FlowJo software (Tree Star, Inc.). Transduction efficiency was measured by calculating transduction index ($TI = \% GFP^+ \text{ cells} \times \text{geometric mean fluorescence intensity [GMFI]}$).

AAV transduction was initially assessed by screening serotypes 1–9, with a multiplicity of infection (MOI) of 5×10^3 viruses per cell. Optimization of AAV2 and AAV3 transduction was performed at MOI values of 5×10^3 , 1×10^4 , 2.5×10^4 , and 5×10^4 . Additionally, single-stranded (ssAAV) and double-stranded (dsAAV) vectors were compared.

Helper-dependent adenovirus transduction was performed with MOIs of 10^1 to 10^4 (calculated as viral particles per cell), which span the typical range of adenoviral MOI values.

MTT assay for cell viability

To assess cell viability through metabolic activity after viral transduction, VICs seeded on a 96-well plate at a density of 40,000 cells per well were transduced at MOIs of 1×10^4 , 2.5×10^4 , and 5×10^4 . Forty-eight hours post-transduction, medium was replaced with fresh medium containing 10% thiazolyl blue tetrazolium (0.5 mg/mL; Sigma) and incubated for 3 h at 37°C. A negative control consisted of untreated cells exposed to 0.1% Triton X-100 (Roche) for 3 min before adding the MTT reagent. Media was replaced with 0.1 N HCl in isopropanol (Sigma) and incubated at room temperature for 15 min to dissolve the formazan crystals. Absorbance measurements were read at 570 nm and subtracted by absorbance at 690 nm using a Spectramax M2 microplate reader (Molecular Devices).

VIC phenotype

VIC phenotype was measured through expression of cytoskeleton marker α -smooth muscle actin (α -SMA). VICs were seeded in 24-well plates as described above and transduced with dsAAV3 (MOI = 10^4). Forty-eight hours post-transduction, cells were harvested by trypsinization, fixed with paraformaldehyde, and permeabilized with methanol. α -SMA expression was measured with flow cytometry after immunostaining with 1:100 dilution of anti-SMA (ab7817; Abcam) and 1:1000 dilution of anti-MS conjugated with AlexaFluor 647 (Life Technologies). For the negative control, mouse IgG (Vector Labs) served as the primary antibody.

Nonviral transfection assay

Transfection with Lipofectamine LTX (Life Technologies), a cationic lipid formulation modified for primary cell transfection, was performed according to manufacturer's instructions using 200 ng plasmid DNA with LTX reagent volumes of 0.4, 0.7, and 1 μ L. Permissive HEK293T cells were used as a positive control. VICs were seeded on 48-well plates at a density of 6.0×10^4 cells per well, 20 h before transfection. HEK293T cells were seeded at 1.7×10^5 cells per well. Transfection with linear PEI (MW 25,000; Polysciences, Inc.) was performed at various nitrogen to phosphate (NP) ratios (15, 20, and 30) and 500 ng DNA with VICs seeded on 12-well plates, at seeding densities of 1.5×10^5 VICs per well and 5.5×10^5 293T per well. Twenty hours post-transfection, media was replaced with fresh media. Forty-eight hours post-transfection, cells were harvested for flow cytometry analysis. Plasmids used for transfections were purified with Zyppy Plasmid Miniprep Kit (Zymo Research).

Heparin competition assay

VICs were prepared and analyzed as described in AAV Transduction Assay subsection above with the addition of an incubation step before transduction: virus at an MOI of 1.5×10^4 was incubated in various concentrations (log-fold increments from 0 to 5 mg/mL) of soluble heparin (Sigma) in serum-free medium for 1 h at room temperature. Forty-eight hours post-transduction, cells were harvested and analyzed for gene expression using flow cytometry.

AAV internalization assay

Internalization of AAV in VICs was assessed by measuring viral genomes using qPCR. Untreated cells were used as a control. For each experimental group, VICs were seeded in 10 wells on 24-well plates with 80,000 cells per well. After 24 h, virus was added to cells in serum-free media at an MOI of 10^4 . Four hours post-transduction, cells were harvested by trypsinization and centrifugation, then washed twice with PBS, and pooled together. DNA was extracted with EZNA Tissue DNA kit (Omega). Viral genomes were measured using qPCR with primers targeting the CMV promoter.

Statistical analysis

The results of flow cytometry measurements are shown as mean $\pm$ standard error of the mean (SEM). Data were analyzed using GraphPad Prism and R. Statistical analysis was performed with one-way ANOVA with *post hoc* pairwise comparisons using Bonferroni adjusted *t*-tests. A *p*-value less than 0.05 was considered statistically significant.

Results

Transduction of primary VICs by different AAV serotypes

To determine whether any AAV serotypes, or variants, are capable of efficiently transducing VICs, we performed *in vitro* transduction assays using primary VICs obtained from porcine aortic valves with nine different AAV serotypes (1–9) packaging a GFP transgene. VICs were incubated for 16 h with the viruses (MOI of 5×10^3 viruses per cell) and harvested 48 h post-transduction to measure GFP expression with flow cytometry (Fig. 1). Gene delivery efficiency was quantified by calculating the TI, the percentage of GFP-positive cells (% GFP⁺) multiplied by the GMFI. The TI parameter is a linear indicator of viral transduction capacity and, thus, is better than the % GFP⁺ cells parameter alone, which saturates quickly with increased MOIs.²²

Of the serotypes tested, AAV serotype 2 (AAV2) and serotype 3 (AAV3) are most effective. While the AAV3 transduction efficiency is higher than AAV2, the difference is not statistically significant ($p=0.34$). AAV5 and AAV6 also demonstrate GFP expression levels above background, but their TI values are substantially lower (5–14 $\times$ lower) than AAV2 and AAV3 ($p<0.01$ for all comparisons). The GMFI of AAV5 and AAV6 are similar to the GMFI of AAV2 and AAV3, however, the % GFP⁺ cells are much lower in AAV5 and AAV6, leading to lower TI values. None of the other serotypes tested lead to gene delivery levels above the untreated cells. Additionally, AAV cytotoxicity was assessed with an MTT assay for mitochondrial activity. Viabilities of VICs exposed to viruses at all tested MOIs are not statistically different than untreated cells (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tec). Moreover, AAV transduction

FIG. 1. Transduction efficiency of adeno-associated virus (AAV) serotypes 1–9 in primary valvular interstitial cells (VICs). VICs were transduced at a multiplicity of infection (MOI) of 5000 with AAV serotype variants packaging *GFP* and analyzed with flow cytometry to measure (a) percentage of GFP-positive (% GFP⁺) cells, (b) geometric mean fluorescence intensity (geom. MFI), and (c) transduction index (TI). AAV2 and AAV3 are the most efficient transducing vectors. U indicates untreated group. Error bars indicate the standard error of the mean (SEM) of four independent experiments. Significant differences compared to untreated: * $p<0.05$, *** $p<0.001$. GFP, green fluorescent protein.

FIG. 2. Optimization of AAV2 transduction in VICs. Single-stranded AAV2 (ss) and double-stranded, self-complementary AAV2 (ds) were compared at a range of MOI values. Flow cytometry was used to quantify (a) % GFP⁺ cells, (b) geom. MFI, and (c) TI. Transduction efficiency is improved with dsAAV2 compared to ssAAV2. U indicates untreated group. Error bars indicate SEM from three and two independent experiments for ssAAV2 and dsAAV2, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

has no effect on VIC phenotype, as measured by α -SMA expression ($p = 0.7$, Supplementary Fig. S2). α -SMA expression in both untreated and treated VICs is within the typical range (50–65%) for porcine VICs cultured on plastic.⁴ In summary, the results thus far demonstrate AAV2 and AAV3 transduce VICs more efficiently than other serotypes.

Optimization of AAV2 and AAV3 transduction efficiencies in VICs

To determine the maximum transduction output of AAV2 and AAV3 in VICs, a transduction assay was performed using a range of increasing MOIs. The transduction profile of single-stranded AAV2 (ssAAV2) increases as MOI increases from 5×10^3 to 5×10^4 (Fig. 2), reaching up to 45% of the cell population expressing GFP.

AAV transduction has been shown to be more efficient and rapid with self-complementary, or double-stranded, genomes versus single-stranded genomes.²³ Single-stranded genomes require a rate-limiting step of second-strand synthesis before transcription occurs. In contrast, self-complementary genomes bypass second-strand synthesis by self-annealing to form double-stranded molecules. Thus, we sought to optimize transduction in VICs by using self-complementary vectors. Using dsAAV2, the % GFP⁺ cells plateaus at an MOI of

2.5×10^4 , but GMFI continues to increase as MOI increases. As such, the TI increases with MOI. Compared to ssAAV2, dsAAV2 results in a 2.6-fold increase in TI and 2.2-fold increase in % GFP⁺ cells (98%) at an MOI of 5×10^4 .

AAV3 transduction is similarly dependent on the MOI and ssAAV versus dsAAV vectors (Fig. 3). ssAAV3-mediated gene delivery results in a maximum GFP expression in 38% of the cell population. In contrast, self-complementary AAV3 transduces 90% of cells and displays a 3.1-fold increase in TI. The transduction levels achieved with AAV2 versus AAV3 are comparable across the range of MOIs tested and with ssAAV and dsAAV vectors. These results show that dsAAV2 and dsAAV3 promote efficient transduction of VICs in higher levels than ssAAV vectors.

AAV-mediated gene delivery was compared to adenoviral vector gene delivery and nonviral transfection methods. Helper-dependent adenoviral gene delivery at an MOI of 10^4 is of moderate efficiency, resulting in 53% GFP⁺ cells (Supplementary Fig. S3). With additional optimization, adenovirus may also be a very effective vector for gene delivery to valvular cells. However, adenoviral vectors are recognized to exhibit substantial adverse immunogenicity,^{24–27} which may need to be addressed depending on the needs of the application. In comparison to AAV-mediated gene delivery, Lipofectamine LTX and PEI demonstrates

FIG. 3. Optimization of AAV3 transduction in VICs. Single-stranded AAV2 (ss) and double-stranded, self-complementary AAV2 (ds) were compared at a range of MOI values. Flow cytometry was used to quantify (a) % GFP⁺ cells, (b) geom. MFI, and (c) TI. Transduction efficiency is improved with dsAAV3 compared to ssAAV3. U indicates untreated group. Error bars indicate SEM from three independent experiments for ssAAV3 and dsAAV3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIG. 4. AAV2 and AAV3 transduction of VICs is heparan sulfate proteoglycan (HSPG)-dependent. AAV2 and AAV3 were added to VICs (MOI=15,000) in the presence of increasing concentrations of soluble heparin. Flow cytometry analysis of (a) % GFP⁺ cells and (b) normalized transduction index (nTI), calculated by normalizing values to the 0 mg/mL heparin condition, show decreasing GFP expression with increasing heparin concentrations. Error bars represent SEM of three and two independent experiments for AAV2 and AAV3, respectively.

substantially lower gene delivery efficiencies in VICs, yielding 3% GFP⁺ cells using Lipofectamine LTX and 1% GFP⁺ cells using PEI (Supplementary Fig. S4).

Heparan sulfate proteoglycan-dependent transduction

AAV2 and AAV3 both use heparan sulfate proteoglycans (HSPG) as their primary cell receptor.^{28,29} Thus, a heparin competition assay was performed to verify whether AAV2 and AAV3 transduction of VICs are also HSPG-dependent as expected (Fig. 4). Before transduction, AAV2 and AAV3 were incubated with increasing concentrations of soluble heparin for 1 h. For AAV2, transduction efficiency dropped to baseline levels with 0.05 mg/mL heparin ($p < 0.01$), whereas 0.5 mg/mL heparin was necessary to reduce AAV3 to baseline levels ($p < 0.01$). In summary, these observations demonstrate AAV2 and AAV3 transduction of primary VICs *in vitro* are HSPG-dependent.

AAV internalization in VICs

One obstacle of gene delivery is vector internalization into target cells. Differences in AAV transduction efficien-

cies may be explained by the ability of various serotypes to enter target cells. AAV internalization was measured by quantifying viral genomes from total cell DNA extractions of transduced VICs. AAV2 and AAV3, which had the highest transduction efficiencies, are internalized over 3 log-fold greater than AAV9 at the same MOI, although the differences are not statistically different ($p = 0.13$ and $p = 0.1$, Fig. 5). These results suggest that AAV2 and AAV3 are internalized by VICs more efficiently than AAV9 (and likely other serotypes), leading to enhanced transgene expression.

Discussion

This study presents AAV as an efficient vehicle for gene delivery to VICs. Different AAV serotypes were compared and optimized for transduction efficiency. The data show AAV2 and AAV3 are capable of efficiently transducing primary porcine VICs *in vitro*. Furthermore, results from the soluble heparin competition assay suggest that these serotypes bind and enter VICs via HSPG, which comprise 3–7% of total glycosaminoglycan content in adult aortic valves.³⁰ The similar transduction profiles of AAV2 and AAV3 may be explained by their high level of homology: AAV2 and AAV3 share 82% DNA sequence homology and 87% protein sequence homology of capsid proteins.³¹ The primary cell receptor for both AAV2 and AAV3 is HSPG^{28,29} and a recent study suggests that AAV2 binds 6-O and N-sulfated heparin while AAV3 binds 2-O and N-sulfated heparin.³² AAV2 and AAV3 are reported to bind the same co-receptors, including human fibroblast growth factor receptor,^{33,34} hepatocyte growth factor receptor,^{35,36} and laminin receptor.³⁷ AAV2 also binds $\alpha V\beta 5$ integrin.³⁸ Although AAV2 and AAV3 have high homology and share common cellular receptors, several studies show that AAV2 displays a broad tropism while AAV3 is poor at transducing most cells and tissues.³⁹ Future studies will be needed to elucidate the mechanisms behind cellular internalization of AAV2 and AAV3 into VICs.

AAV is the most effective gene delivery vehicle for VICs compared to synthetic vectors Lipofectamine LTX and PEI. Recombinant viral vectors are generally efficient because viruses have evolved in nature to circumvent several barriers to gene delivery that include cell binding and uptake, endosomal escape, and nuclear localization. On the other hand,

FIG. 5. Internalization of AAV vectors in VICs. dsAAV2, dsAAV3, and dsAAV9 were added to VICs (MOI=10⁴) for 4 h, then harvested for extraction of genomic DNA. Viral genomes were determined with quantitative polymerase chain reaction (qPCR). Values are reported as viral genomes normalized to total mass of DNA. AAV2 and AAV3 vectors were internalized 3 log-fold more than AAV9. U indicates untreated group. Error bars represent SEM of three independent experiments.

synthetic vectors may be unsuccessful at gene delivery if they lack the means to navigate this series of obstacles.⁴⁰

It is difficult to compare quantitatively the results of this study to previous findings reported in the literature as few studies present quantitative functional gene delivery to VICs. siRNA delivery by Lipofectamine and N-TER were reported to reach ~90–100% of VICs, measured by the presence of nontargeting fluorophore-labeled siRNA.^{5,13} However, the efficiency of siRNA uptake may be misleading because it does not correlate with functional siRNA-mediated RNA interference.⁴¹

Gene delivery by ssAAV involves a rate-limiting step of second-strand synthesis before transgene expression can occur. A self-complementary AAV construct overcomes this limitation because it is designed to self-assemble into a double-stranded form.^{23,42} A double-stranded construct has the benefits of faster onset of transgene expression and higher transduction efficiency, which are critical features when culturing VICs within the limit of five passages. Moreover, we show that the percentage of cells transduced by dsAAV plateaus at a lower MOI than by ssAAV, and the transduction efficiency of dsAAV is about three-fold greater than that of ssAAV. However, scAAV has the drawback of half the packaging capacity of ssAAV (~2.3 kb vs ~4.7 kb).⁴³ This limit does not preclude scAAV from being used to deliver shRNA or microRNA. Small protein-coding cDNA such as transforming growth factor- β 1 (TGF- β 1) may also be accommodated. Gene delivery of TGF- β 1 could facilitate future research of the cytokine's role in valve disease, which has thus far shown to induce myofibroblast activation, pathological calcific phenotypes, and glycosaminoglycan elongation that corresponds with increased lipid binding in early phases of calcific aortic valve disease.^{4,44–46} Additionally, AAV may be used as an efficient vehicle to deliver CRISPR/Cas systems for genome engineering with precise and multiplexed gene targeting.⁴⁷

The findings reported here suggest AAV is a promising tool for *in vitro* gene delivery, which will be valuable for *ex vivo* applications where valve cells are genetically manipulated before seeding tissue engineering scaffolds or reinjection into the body. To carry out *in situ* genetic manipulation of valve cells, however, further studies are necessary to confirm the efficacy of *in vivo* gene delivery with AAV to valvular cells. Numerous barriers to gene delivery, including adverse interaction with blood components and target tissue specificity, will greatly impact the potential applicability of AAV vectors via *in vivo* administration.

In conclusion, scAAV2 and scAAV3 are effective vectors for gene delivery to primary VICs. By optimizing the MOI used and packaging self-complementary genomes, high levels of gene delivery can be achieved under *ex vivo* experimental conditions. In the future, the genetically manipulated VICs can be used to test the efficacy of gene therapies, incorporated into tissue-engineered valve constructs, or used to study basic questions regarding the pathophysiology of valve diseases.

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Disclosure Statement

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