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A three-dimensional co-culture model of the aortic valve using magnetic levitation

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ABSTRACT

The aortic valve consists of valvular interstitial cells (VICs) and endothelial cells (VECs). While these cells are understood to work synergistically to maintain leaflet structure and valvular function, few co-culture models of these cell types exist. In this study, aortic valve co-cultures (AVCCs) were assembled using magnetic levitation and cultured for 3 days. Immunohistochemistry and quantitative reverse-transcriptase polymerase chain reaction were used to assess the maintenance of cellular phenotype and function, and the formation of extracellular matrix. AVCCs stained positive for CD31 and α-smooth muscle actin (αSMA), demonstrating that the phenotype was maintained. Functional markers endothelial nitric oxide synthase (eNOS), von Willebrand factor (VWF) and prolyl-4-hydroxylase were present. Extracellular matrix components collagen type I, laminin and fibronectin also stained positive, with reduced gene expression of these proteins in three dimensions compared to two dimensions. Genes for collagen type I, lysyl oxidase and αSMA were expressed less in AVCCs than in 2-D cultures, indicating that VICs are quiescent. Co-localization of CD31 and αSMA in the AVCCs suggests that endothelial–mesenchymal transdifferentiation might be occurring. Differences in VWF and eNOS in VECs cultured in two and three dimensions also suggests that the AVCCs possibly have anti-thrombotic potential. Overall, a co-culture model of the aortic valve was designed, and serves as a basis for future experiments to understand heart valve biology.

1. Introduction

The aortic valve, located between the left ventricle and the aorta, regulates unidirectional blood flow from the heart to the systemic circulation. The valve consists of three leaflets, each of which is comprised of two cell types: valvular interstitial cells (VICs) that populate the interior of the leaflet, and valvular endothelial cells (VECs) that cover the surface of the leaflet. VICs are a heterogeneous group of cells with fibroblast and smooth muscle cell phenotypes that maintains the extracellular matrix (ECM) of the leaflet [1–3]. VECs regulate the transfer of signals in the bloodstream to the valve interior, mediate inflammatory and hemostatic responses, and have a phenotype similar to vascular endothelial cells, but demonstrate major differences in alignment to flow and mechanobiology [4–7]. Together, both VICs and VECs work to maintain the leaflet structure and valve function.

The interactions between these two cell types are critical for normal valve function. The presence of VECs has been shown to keep VICs in a quiescent state, as defined by the low expression of α-smooth muscle actin (αSMA) [8]. The key role that their interaction plays in valve function is also demonstrated by the finding that dysfunction of both cell types appears in calcific aortic valve disease (CAVD) [9]. Injury to the valvular endothelium leads to thrombosis, inflammation and lipid accumulation [10–15]. These factors lead to the activation of VICs, or increased expression of αSMA, matrix remodeling, and their progression towards an osteoblastic phenotype, which ultimately result in calcification and stenosis [16–20].

Despite the well-established notion that both cell types and the interaction between the two are critical to valve maintenance, function and disease, there are few co-culture models of VECs and VICs in the literature. The lack of co-culture models of the aortic valve can partly be attributed to the inability to produce such models using traditional 2-D cell culture techniques, with which the majority of research on valvular cells is conducted. 2-D environments are poor representations of the native 3-D environment

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in which valvular cells reside [21–23]. The inadequacy of 2-D cultures for valvular research is highlighted by the varied results of studies on the effect of statins as treatments for calcific aortic valve disease. Statins in 2-D *in vitro* cultures of VICs significantly reduced calcific nodule size and area, as well as αSMA expression [24–28], yet clinical trials have not shown any improvement in outcome for patients with calcific aortic stenosis who took statins [29–33]. Indeed, studies of the effect of statins on VICs in 3-D collagen gels showed a smaller, conditional reduction in calcification in comparison to 2-D cultures [26]. These results demonstrate the enormous gap in complexity and fidelity between simple 2-D cell culture models and the human body, and necessitate the development of cost-effective, clinically relevant and representative 3-D co-culture models of the aortic valve.

To that end, this study used a magnetic levitation method with magnetic nanoparticles to assemble 3-D co-cultures of VECs and VICs. In this method, cells are incubated with a nanoparticle assembly consisting of poly-L-lysine, magnetic iron oxide (MIO; Fe_3O_4, magnetite) and gold nanoparticles that form a gel via electrostatic interactions [34–37]. The uptake of this gel by cells renders them magnetic and allows for their manipulation, specifically by levitating the cells off the surface into the media, where the cells aggregate and interact to form larger 3-D structures. This method has previously been used to create 3-D cultures of glioblastomas, smooth muscle cells, adipose stem cells and pulmonary cells [37–41]. Magnetically levitated human glioblastoma cells demonstrated greater proliferation and more *in vivo*-like protein expression in comparison to 2-D cultures [37].

In addition, this method has previously been used to create co-cultures. Layered co-cultures of the lung were sequentially assembled using epithelial cells, smooth muscle cells, fibroblasts and endothelial cells within 8 h [41]. After 2 days of culture, ECM (collagen type I, fibronectin, laminin) was formed and organized, and the phenotypes of all four cell types were maintained. After 7 days, epithelial cell function and phenotype were still present [41]. In comparison, previously reported co-culture models of the valve leaflet consisted of collagen-based gels which were embedded and contracted with VICs, and then seeded with VECs on their surfaces in a process that took between 4 and 8 days to assemble [8,42]. As a result, magnetic levitation is an alternative method for rapidly assembling co-culture models.

Based on the success of the magnetically levitated co-culture of the bronchiole, this study used magnetic levitation to create 3-D co-cultures of aortic valve cells. VICs and VECs were sequentially assembled into layered co-cultures. Immunohistochemistry was used to verify the phenotype and function of both VECs and VICs, and assess ECM formation within the co-culture. Unlike previous studies using magnetic levitation, this study is the first to analyze the gene expression profiles of these cultures using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). In addition, the effects of the magnetic nanoparticles and exposure to the magnetic field on cell proliferation were investigated. The expected result of this study was a VIC and VEC co-culture model that maintained cell phenotype and function, and synthesized relevant ECM.

2. Materials and methods

2.1. Cell isolation and culture

Aortic valves were extracted from fresh porcine hearts obtained from a local commercial abattoir (Fisher Ham and Meats, Spring, TX). Aortic VECs and VICs were harvested as previously described [43,44]. Both cell types were cultured in an incubator (37 °C, 5% CO_2, 95% humidity) with changes of medium every other day.

VECs were isolated from the leaflets via digestion using collagenase II (60 U ml^{-1}) and dispase (2 U ml^{-1}) [43]. VECs were seeded on flasks or glass slides coated with 2.5% gelatin in 1:1 H_2O:phosphate-buffered saline (PBS, pH ~7.4) [45], and cultured in specialized medium (EGM-2, Lonza Biosciences, Walkersville, MD) with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). At the first passage, magnetic cell sorting was used to purify the VECs for CD31+ cells (mouse monoclonal anti-CD31 antibody TLD-3A12, Millipore, Billerica, MA) [43]. VECs were used at their third passage.

VICs were isolated from the leaflets with a multistep digestion using collagenase II (~450 U ml^{-1}), hyaluronidase (~50 U ml^{-1}) and collagenase III (~350 U ml^{-1}) [44]. VICs were seeded on uncoated flasks or glass slides in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, UT), 1% HEPES buffer and 1% P/S. VICs were used at their third passage.

2.2. Magnetic levitation

Magnetic levitation using the Bio-Assembler Kit (Nano3D Biosciences, Houston, TX) was employed to create 3-D cultures (Fig. 1B) [37]. Confluent flasks of cells were treated with a magnetic nanoparticle assembly (8 μl cm^{-2} of cell culture surface area or 50 μl ml^{-1} medium, NanoShuttle (NS), Nano3D Biosciences) for overnight incubation to allow for cell binding to the nanoparticles. NS was fabricated as previously described, by mixing Au nanoparticles prepared by citrate reduction, poly-L-lysine and iron oxide [34,35,37]. Treated cells were then detached with trypsin and resuspended in an ultra-low attachment 24-well plate with 400 μl of medium. A magnetic driver of 24 neodymium magnets (field strength = 50 G) designed for 24-well plates and a plastic lid insert were placed atop the well plate to levitate the cells to the air–liquid interface.

2.3. Cell metabolism

The effects of both the NS and magnetic field on cell metabolism over 8 days were measured using an MTT assay. Briefly, MTT reagent (0.5 mg/ml in medium, thiazolyl blue tetrazolium bromide, Sigma–Aldrich, St Louis, MO) was added to each well. After 3–4 h of incubation, the medium was aspirated to yield the formazan blue crystals at the bottom of the well. Acidified isopropanol (0.1 N HCl in isopropanol) was added to dissolve the formazan blue crystals, and the absorbance of the resulting solution was read in triplicate on a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA) at 570 nm with background subtraction at 690 nm. All measurements were normalized to day 0 results.

To test the effects of adding NS (+NS) or exposure to a magnetic field (+mag) to VIC and VEC proliferation, 25,000 cells were seeded in 24-well plates. For +NS wells, NS was added to the wells (8 μl cm^{-2}) on day 0 for incubation overnight. For +mag wells, neodymium magnets were placed 1 mm underneath each well (field strength = 300 G). Medium was changed at day 1 and every other day after that for the duration of the study. Cell metabolic activities were measured on days 2, 4, 6 and 8 ($n = 9$).

2.4. Co-culture assembly

Magnetic levitation was used to create co-cultures of VICs and VECs (Fig. 2A) [41]. VICs and VECs were incubated overnight with NS, and then levitated into 3-D cultures of 500,000 cells each. After 4 h of levitation, a 0.1875 in. outside diameter Teflon pen housing a neodymium magnetic rod was used to sequentially pick up a 3-D culture of each cell type to assemble the co-culture: first VECs, then VICs. The still attached co-culture was then submerged in 150 μl

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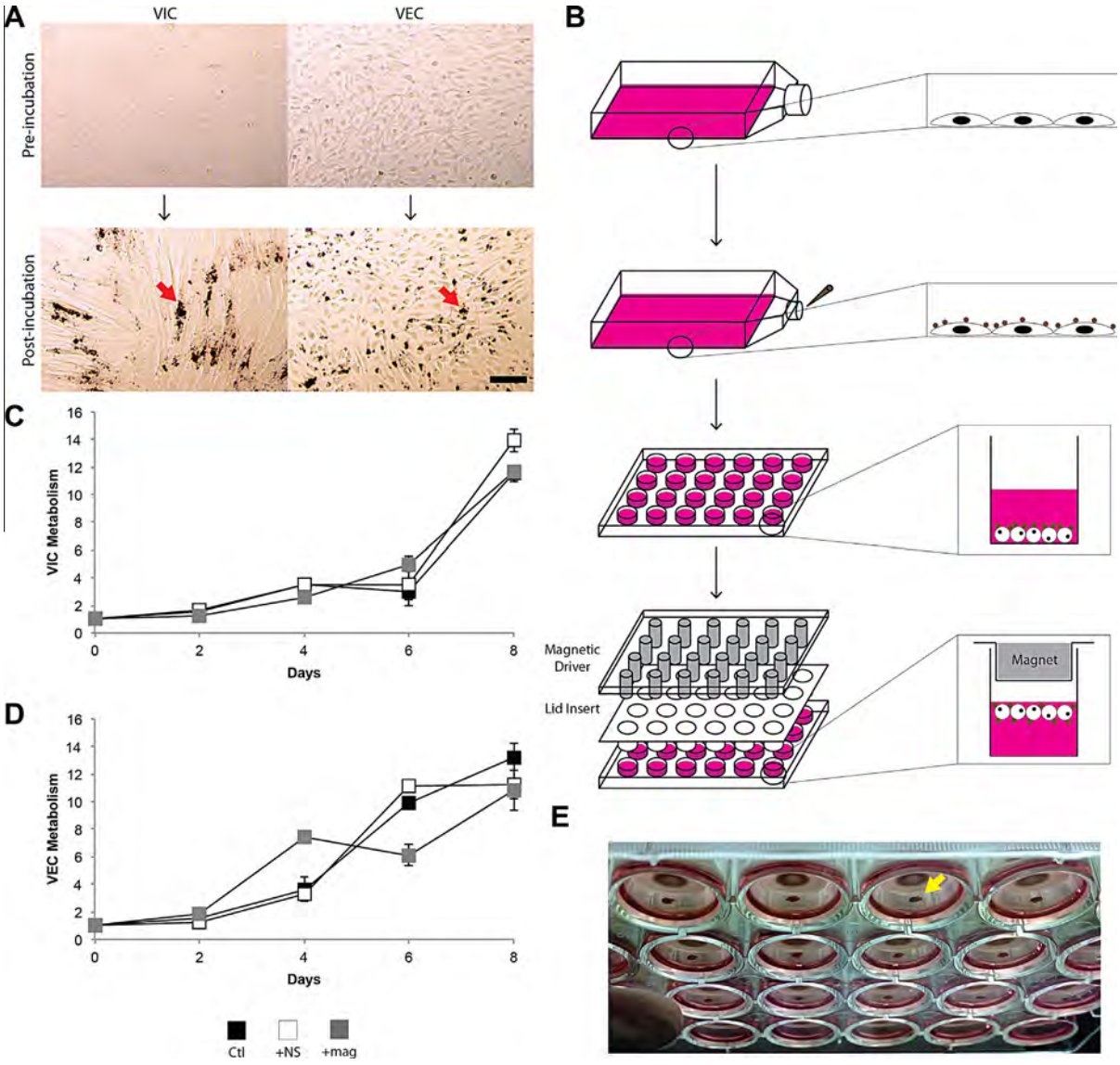

Fig. 1. (A) VICs and VECs before and after incubation with NanoShuttle. Note the maintenance of morphology after incubation, and the localization of the magnetic nanoparticles (indicated by red arrow) with the cells. Scale bar = 100 μm. (B) Schematic of magnetic levitation. A confluent flask of cells is incubated with NanoShuttle overnight to allow for cell binding. The next day, the cells are detached from the surface and resuspended in 400 μl of medium in a 24-well plate. A magnetic driver and plastic lid insert is placed on top of the well plate to levitate the cell constructs to the air–liquid interface. Metabolic activity of VICs (C) and VECs (D) with incubation with NanoShuttle and exposure to the magnet over 8 days ($n = 9$). There was no significant difference found between these groups for both cell types. Error bars represent standard error of the mean. (E) 3-D cultures (indicated by yellow arrow) levitating in a 24-well plate as seen from below the plate.

VEC medium in a 96-well plate for 4 h. The co-culture was then put back into a 24-well plate by first filling a well with 400 μl of VEC medium, then removing the rod magnet from the Teflon pen, and placing the magnetic driver underneath the well to attract the co-culture into the well. Once detached, the magnetic driver was moved to the top of the well and the co-culture was levitated again. These co-cultures are hereinafter referred to as the aortic valve co-culture (AVCC).

2.5. Immunohistochemistry

Immunohistochemistry (IHC) was used to verify the maintenance of phenotype and function, and the formation of ECM. AVCCs were fixed in 4% paraformaldehyde for at least 5 h, then dehydrated, embedded in paraffin and sectioned according to standard procedures. To preserve the structure of the AVCCs, the Teflon pen used for co-culture assembly was used to hold the co-culture in place and maintain its structure during processing. For 2-D immunocytochemistry (ICC), VICs and VECs were seeded on glass chamber slides at 50,000 cells per well. The next day, cells were fixed with 4% paraformaldehyde.

Sections to be stained were rehydrated and underwent antigen retrieval using a citrate buffer solution (Antigen Decloaker, Biocare Medical, Concord, CA) at 80 °C for 30 min. For intracellular antigens, sections were then permeabilized using 0.2% Triton X-100 for 15 min. All sections were then washed and blocked using 1% donkey serum (GeneTex, Irvine, CA) in PBS for 1 h at room temperature. Experimental sections were then incubated overnight at 4 °C with the primary antibody of interest at the manufacturer's recommended dilution in PBS with 1% bovine serum albumin. Negative controls were left incubating with donkey serum. The next day, all sections were washed and incubated with a fluorescent secondary antibody (AlexaFluor 488/633, Invitrogen, Carlsbad, CA) for 1 h at room temperature and counterstained with DAPI

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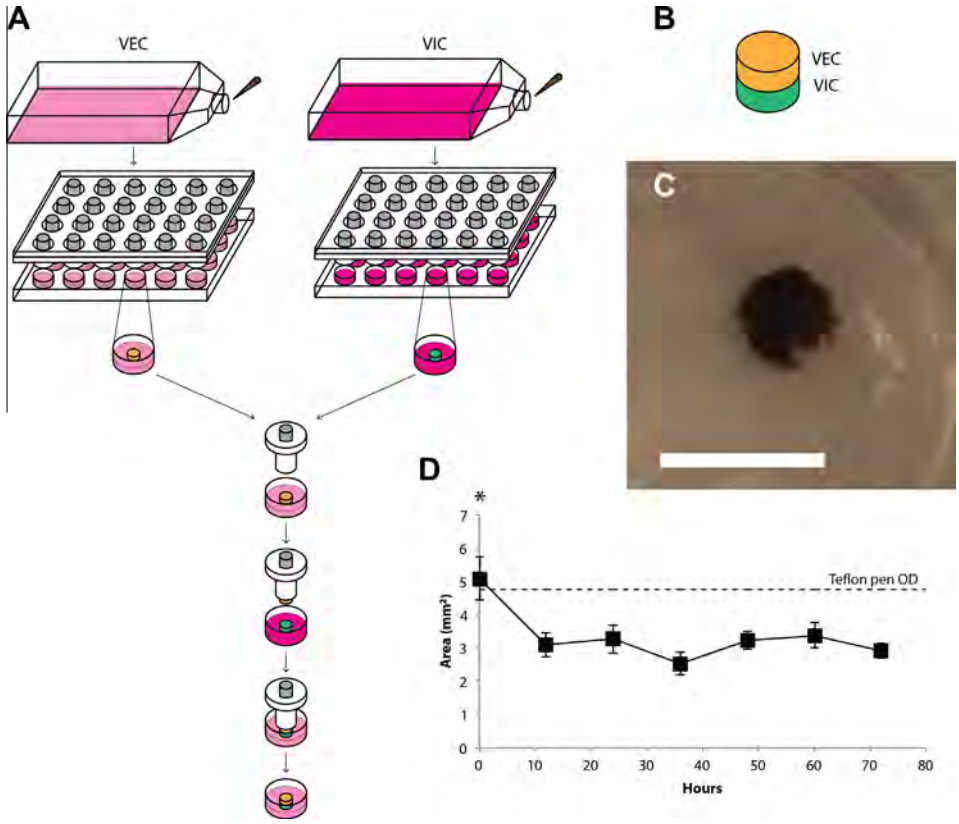

Fig. 2. (A) Schematic of co-culture assembly using magnetic levitation. 3-D cultures of VECs (yellow) and VICs (green) were separately levitated for 4 h. A Teflon pen was used to then sequentially assemble the co-culture together: first VECs, then VICs. The co-culture is submerged in medium, still attached to the Teflon pen, for 4 h. The co-culture is then detached from the Teflon pen and levitated for 3 days in VEC medium. (B) Schematic of the co-culture, within which the VEC layer sits on top of the VIC layer. (C) A resulting VIC–VEC culture after 3 days of levitation as seen from above the plate. Scale bar = 5 mm. (D) The planar size of the co-culture over 3 days ($n = 9$). The co-culture starts close to the size of the Teflon pen used to assemble the co-culture. By 12 h, the co-culture significantly shrinks in size and maintains that size over the next 60 h. $^{*}P < 0.05$ vs. the rest.

(KPL, Gaithersburg, MD) for 15 min. All slides were then washed, mounted and imaged. 2-D ICC slides were stained similarly to 3-D cultures after permeabilization with Triton X-100. Images were captured on a confocal microscope (LSM 510 META NLO, Zeiss).

The antigens stained for in this study included: αSMA (Abcam, Cambridge, MA) for VIC phenotype; CD31 (Abcam) for VEC phenotype; collagen type I (Col I, Abcam), laminin (Lam, Abcam) and fibronectin (FN, Abcam) for ECM; prolyl 4-hydroxylase (P4H, Bioss, Woburn, MA) for collagen synthesis; endothelial nitric oxide synthase (eNOS, Santa Cruz Biotechnology, Santa Cruz, CA) and von Willebrand factor (VWF, Abcam) for endothelial function; and VE-cadherin (VE-cad, Cell Signaling Technology, Danvers, MA) and N-cadherin (N-cad, Invitrogen) for cell–cell interactions. For each antigen, the AVCCs, 3-D and 2-D cultures were stained at the same time. See Supplementary Table S1 for catalog numbers of each antibody.

2.6. Quantitative reverse-transcriptase polymerase chain reaction

Sample mRNA was extracted using Trizol Reagent (Invitrogen) mediated lysis and a series of ethanol washes and centrifugations. The mRNA was reverse-transcribed into cDNA using Primescript 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan). The cDNA samples were stored at −20 °C until use. Using the 2X QuantiTect SYBR Green PCR Master Mix (Clontech Laboratories, Mountain View, CA), qRT-PCR on the cDNA was performed (Mastercycler ep realplex, Eppendorf, Hamburg, Germany) to assess differences in gene expression levels between the AVCC, 3-D VEC and VIC cultures, and 2-D VEC and VIC cultures. For quantification, the GAPDH gene (Integrated DNA Technologies, Coralville, IA) was used as the housekeeping gene, and sample group gene expression levels were normalized to the corresponding expression levels of the AVCCs. The AVCCs were used as the standard for the purposes of ease of analysis and comparison, given that some markers are specific to either VICs or VECs.

qRT-PCR was performed ($n = 3–5$) to measure the positive gene expression of: αSMA for VIC phenotype; CD31 for VEC phenotype; COL1A1, FN, Lam-β1 for ECM; lysyl oxidase (LOX) for collagen fibrillogenesis; and eNOS and VWF for endothelial function (Integrated DNA Technologies) (Table 1).

2.7. Statistical analysis

One-way ANOVAs were performed on the data (JMP, SAS, Cary, NC), with significance defined as $P < 0.05$. If a significant effect was observed, *post-hoc* Tukey's testing was used to observe pairwise comparisons. Data is presented as mean ± standard error of the mean.

3. Results

3.1. Magnetic levitation

Both VICs and VECs were successfully levitated into 3-D cultures. Both VICs and VECs were able to bind with the NS without grossly affecting morphology (Fig. 1A). Neither incubation with

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Table 1
Summary of the DNA primer sequences used to assess sample gene expression using qRT-PCR.

Gene		5′ ← DNA sequence → 3′	Product size	Accession No.
ACTA2 (αSMA)	Forward	AATAGAACACGGCATCATC	77	FJ547477
	Reverse	CACGAAGCTCATTGTAGAA		
PECAM1 (CD31)	Forward	ACTGCTAACAACCAGAATT	80	X98505
	Reverse	GCTTGACAGGAGAATAATATAAC		
COL1A1	Forward	AGTTGTCTTATGGCTATGATGAG	78	XM_003483014
	Reverse	GACCACGAGGACCAGAAG		
FN1	Forward	CTACTATTACTGGTCTGGAA	75	AY839862
	Reverse	CACTCTTCTGATTGTTCTT		
LAMB1	Forward	CACCACGGATTCCAACAG	75	AF329358
	Reverse	TGCTCCAACATCAAGTCT		
LOX	Forward	CAGTGGATTGATATTACAG	99	NM_001206403
	Reverse	ATTGTTGGAATAGTCTGA		
VWF	Forward	CGAACCCAAGAAGAGAAT	108	S78431
	Reverse	ATCACTTCCTCCACAAAC		
NOS3 (eNOS)	Forward	AGAGAATGGAGAGAGTTT	104	AY266137
	Reverse	TATTGAAGCGGATTTTGT		
GAPDH	Forward	CATTGACCTCCACTACAT	119	AF017079
	Reverse	AGATGGTGATGGGATTTC		

NS nor exposure to the magnetic field significantly affected the metabolism of VICs and VECs (Fig. 1C, D).

3.2. Co-culture assembly

AVCCs were successfully assembled and maintained for 3 days. At day 3, the AVCCs still maintained their competent structure (Fig. 2B). After 12 h of levitation, the planar size of these co-cultures decreased significantly, but did not change for the remaining 60 h (Fig. 2C). The AVCCs were approximately 500 μm thick after culture. Hematoxylin and eosin staining showed two color stains, demonstrating the presence of two distinct cell types (see Supplementary Fig. S3).

3.3. Phenotypic markers

Immunohistochemistry demonstrated the presence of VICs and VECs within the AVCCs by positive staining for αSMA and CD31 (Fig. 3A, for negative controls see Supplementary Fig. S4). Both 3-D and 2-D VIC cultures stained positively for αSMA. Similarly, both 3-D and 2-D VEC cultures stained positively for CD31, and both 3-D monotype cultures stained positively for αSMA. qRT-PCR confirmed the IHC results, as AVCCs expressed both αSMA and CD31 mRNA (Fig. 3B). There was no difference in αSMA gene expression between the AVCC and 3-D monotype cultures, but these cultures expressed significantly less αSMA mRNA than 2-D VIC cultures (652.078× vs. AVCC, $P < 0.05$). CD31 gene expression in the AVCCs was statistically similar to that in 3-D monotype cultures and 2-D VEC cultures. There was virtually no CD31 expression by 3-D VIC cultures (0.007× vs. AVCC), and CD31 expression by 3-D VEC cultures was significantly higher than that in 3-D VIC cultures (471.990× vs. 3D VIC, $P < 0.05$), whereas CD31 expression by 2-D VEC cultures trended higher than 3-D VIC cultures (427.439× vs. 3-D VIC, $P = 0.055$).

3.4. Functional markers

All functional markers of interest stained positively in the AVCCs (Fig. 4A, for negative controls see Supplementary Fig. S5). VE-cad was limited to the outer edges of the AVCCs and co-localized with CD31. Similarly, VWF was located towards the edge of the AVCCs. N-cad, P4H and eNOS all stained positively throughout the AVCCs. qRT-PCR demonstrated that LOX gene expression was significantly less in the AVCCs than in both 2-D VIC (14.963× vs. AVCC, $P < 0.005$) and VEC (11.310× vs. AVCC, $P < 0.05$) cultures, but similar to both 3-D VIC and VEC cultures (Fig. 4B). VWF gene expression in 2-D VEC cultures was the highest, and significantly higher than 3-D monotype cultures and the AVCCs (48.571× vs. AVCC, $P < 0.05$). Similarly, eNOS gene expression was highest in 3-D VEC cultures, where it was significantly higher than 3-D VIC cultures, 2-D VEC cultures and the AVCCs (5.606× vs. AVCC, $P < 0.005$).

3.5. Extracellular matrix

With regards to the ECM components of interest, Col I, FN and Lam stained positively, and were evenly distributed within IHC stains of the AVCCs (Fig. 5A, for negative controls see Supplementary Fig. S6). In 2-D cultures, Col I and FN stained more intensely and prominently in 2-D VIC cultures than 2-D VEC cultures, while Lam stain intensity was higher in 2-D VEC cultures. Gene expression for COL1A1 was the highest in 2-D VIC cultures, where it was also significantly higher than the other groups (98.135× vs. AVCC, $P < 0.0001$) (Fig. 5B). There was a significant difference in FN mRNA expression between 2-D cultures and all 3-D cultures (94.967–113.285× vs. AVCC, $P < 0.01$). 2-D VEC cultures expressed significantly higher amounts of Lam-β1 mRNA than 3-D monotype cultures (2.822–5.735× vs. 3D cultures, $P < 0.05$), but statistically similar amounts to 2-D VIC cultures and the AVCCs (2.247× vs. AVCC, $P = 0.095$).

4. Discussion

This study used magnetic levitation to construct a layered co-culture of the aortic valve, the AVCC. This is the first study to both magnetically levitate aortic valve cells, and then use this technique to assemble such co-cultures. AVCCs were assembled and cultured for 3 days in VEC medium. The size of the AVCCs decreased after 12 h of levitation, likely due to cell contraction, then was held constant for the remaining 60 h. Immunohistochemistry was used to stain for markers of cellular phenotype, function and ECM. qRT-PCR was used to quantitatively measure the gene expression for these same markers in these 3-D cultures, the first time it has been used on magnetically levitated cultures. Positive stains and gene expression for αSMA and CD31 verified VIC and VEC phenotype. The presence of P4H, LOX, VWF and eNOS indicates the preservation of function by both cell types. ECM formation in the form of Col I, FN and Lam was demonstrated. The positive stain for N-cad affirmed the cell–cell interactions within the AVCCs, while the positive stain for VE-cad showed that VECs in the AVCCs form tight

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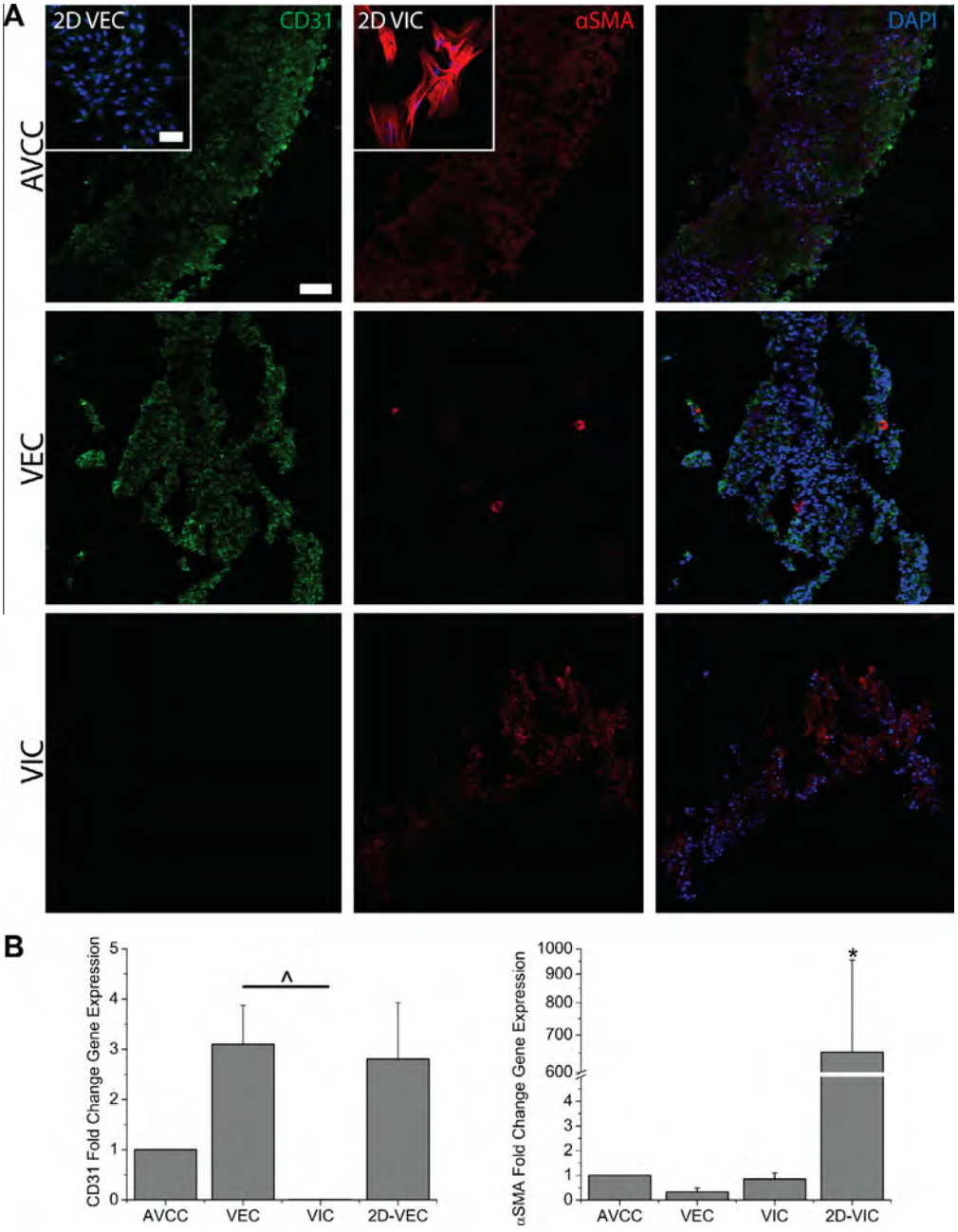

Fig. 3. (A) IHC stains for the phenotypic markers αSMA (red) and CD31 (green) in the AVCC, 3-D VIC and VEC cultures, and 2-D VIC and VEC cultures (insets). Nuclei are counterstained using DAPI (blue). The AVCCs stained positively for both phenotypic markers, indicating the presence of both VICs and VECs. CD31 was limited to the outer edges of the AVCCs, while αSMA was distributed throughout. Scale bar = 50 μm. (B) qRT-PCR results for the phenotypic markers αSMA and CD31 (n = 3–5). αSMA gene expression for the AVCC and 3-D monotype cultures was significantly lower than 2D VIC cultures. CD31 gene expression is non-existent in 3D VIC cultures. $^{*}P < 0.05$ vs. other groups. $^{\wedge}P < 0.05$ within bracket. Error bars represent standard error of the mean.

junctions. This study also affirms the use of magnetic levitation as a simple method to create 3-D co-cultures.

The AVCCs and the 3-D VIC cultures were found to have significantly reduced gene expression of αSMA, as well as COL1A1, FN and P4H, compared to 2-D VIC cultures, suggesting that magnetically levitated VICs in the AVCCs are in a quiescent state [1,46,47]. This quiescence could be a result of the difference in substrate stiffness between 2-D cultures on glass, and 3-D self-aggregated cultures. Higher stiffnesses have previously been shown to increase αSMA expression and the formation of stress fibers [48]. In addition, these cells reverted to a quiescent state from an activated state when levitated from 2-D to 3-D, confirming previous results that VIC activation is both plastic and reversible [47,49]. The presence of VECs could also have reduced VIC activation, as VICs in a collagen-gel based co-culture with VECs demonstrated reduced αSMA expression [8]. This role of VECs is supported by the fact that the gene expressions of COL1A1 and FN were reduced by more than half in AVCCs compared to 3-D VIC cultures, even though VICs made up half of the AVCCs. As an activated VIC state is commonly associated with matrix remodeling and the onset of CAVD [16,17,50], the quiescent state of VICs suggests that the AVCCs can be used to study the activation of VICs under various environmental conditions. Further research is required to understand VIC quiescence and activation within the AVCCs.

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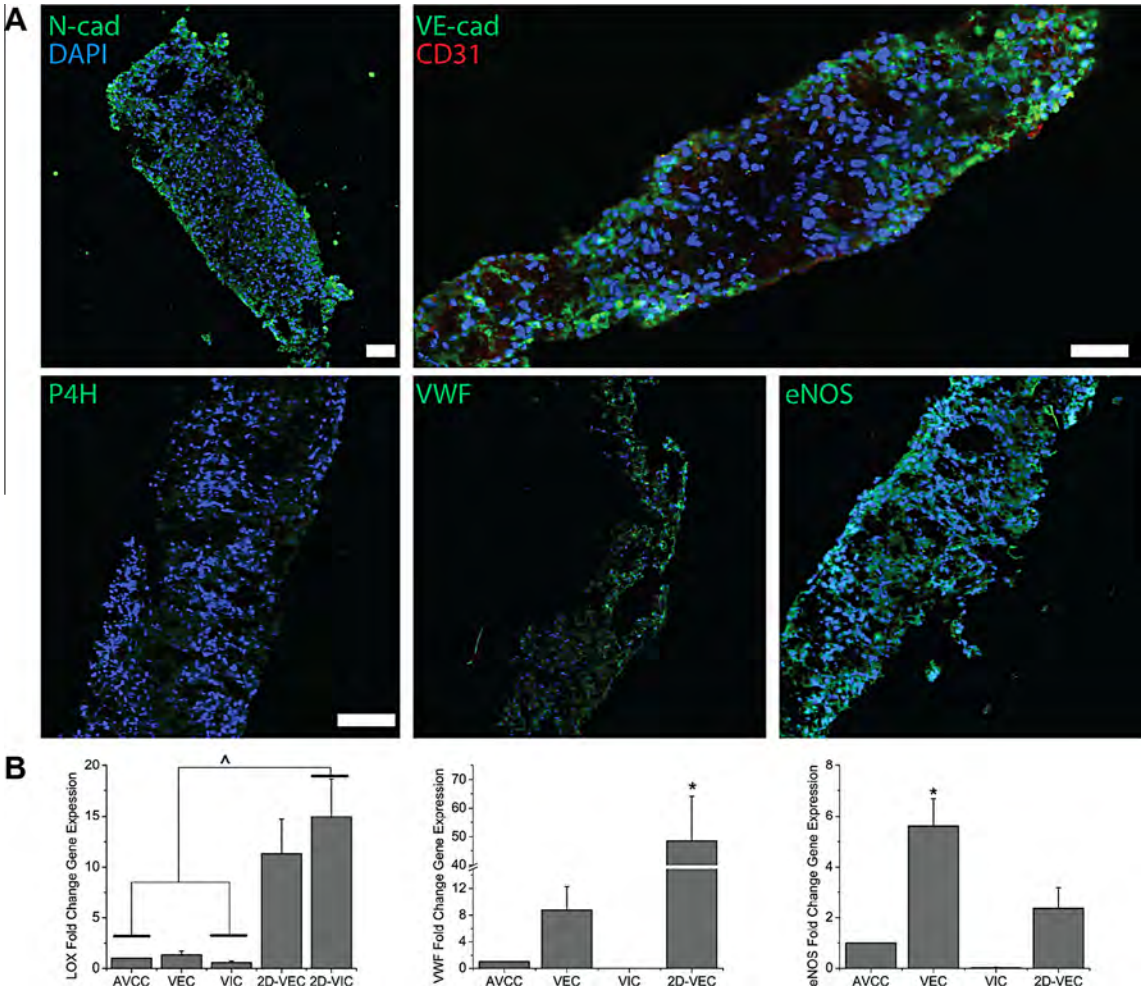

Fig. 4. (A) IHC stains (green) for the functional markers N-cad, P4H, VWF, eNOS and VE-cad (double-stained with CD31 in red) in the AVCC. Nuclei are counterstained using DAPI (blue). The AVCCs stained positively for all these functional markers. VE-cad and VWF were localized to the outer edges of the AVCC, while N-cad, P4H and eNOS were distributed evenly throughout. Scale bar = 100 μm. (B) qRT-PCR results for the functional markers LOX, VWF and eNOS (n = 3–5). Gene expression for LOX and VWF was significantly lower in the AVCCs in comparison to 2-D VIC and VEC cultures, respectively, while for eNOS, expression in the AVCCs was significantly lower than expression in 3-D VEC cultures. $^{*}P < 0.05$ vs. other groups. $^{\wedge}P < 0.05$ within bracket. Error bars represent standard error of the mean.

Interestingly, while VIC phenotype was found to be quiescent and ECM gene expression was significantly less in the AVCCs compared to 2-D cultures, the AVCCs stained positively for ECM components, such as Col I, FN and Lam. The formation and organization of ECM in the AVCCs within 3 days would presumably require VIC activation for matrix remodeling [18]. IHC and qRT-PCR were conducted after 3 days of culture to find a quiescent state, but activation and matrix remodeling could have occurred earlier, yielding the ECM that was observed. Col I was found to have formed in the AVCCs as early as 2 days of levitation (data not shown), while magnetically levitated pulmonary fibroblasts and tracheal smooth muscle cells have been demonstrated to produce and extrude Lam into the extracellular space within 6 h [41]. As with other studies of heart valve biology, αSMA was used alone as a marker of VIC activation [48,51] and to distinguish VICs from VECs within the AVCCs, but other markers for VIC activation, including non-muscle myosin heavy chain (NMM) and embryonic smooth-muscle myosin heavy chain (SMemb) [46,52], should be explored in future studies. Future studies using this model will also require time-based studies to further characterize VIC activation and ECM formation within the AVCCs.

The positive stains for CD31, VWF, eNOS, Lam and VE-cad verify that VEC phenotype and function is maintained within the AVCCs and competent endothelium is being formed. Unexpectedly, on the edges of AVCCs and throughout 3-D VEC cultures, CD31 was co-localized with αSMA. In addition, gene expression for eNOS, which catalyzes nitric oxide in endothelial cells and has previously been used as a functional marker for VECs for its important role in vasoactivity and resistance to oxidative stress [53–55], in the AVCCs was less than half that of 3-D VEC cultures, while consisting of half VECs. Gene expression for CD31, VWF and Lam in the AVCCs also trended similarly to eNOS. Together, these results suggest the possibility of endothelial to mesenchymal transdifferentiation (EnMT) occurring within the AVCCs. VECs commonly differentiate into myofibroblast phenotypes in 2-D *in vitro* culture [43], but the fact that the AVCCs were cultured in VEC media and without a stiff substrate suggest that this result is unique. VECs have been shown to undergo EnMT *in vitro* in a manner stimulated by TGF-β1 and inhibited by vascular endothelial growth factor (VEGF) [56,57]. EnMT has been implicated in valvular disease and mechanically regulated [58], but the finding that the AVCCs were positive for VE-cad, and its VICs were in a quiescent state, would suggest that EnMT is not occurring as a response to injury or endothelial dysfunction within the AVCCs. Rather, VECs could possibly be undergoing EnMT to replenish or supplement VICs, a role previously suggested and investigated as a possible source for progenitor cells

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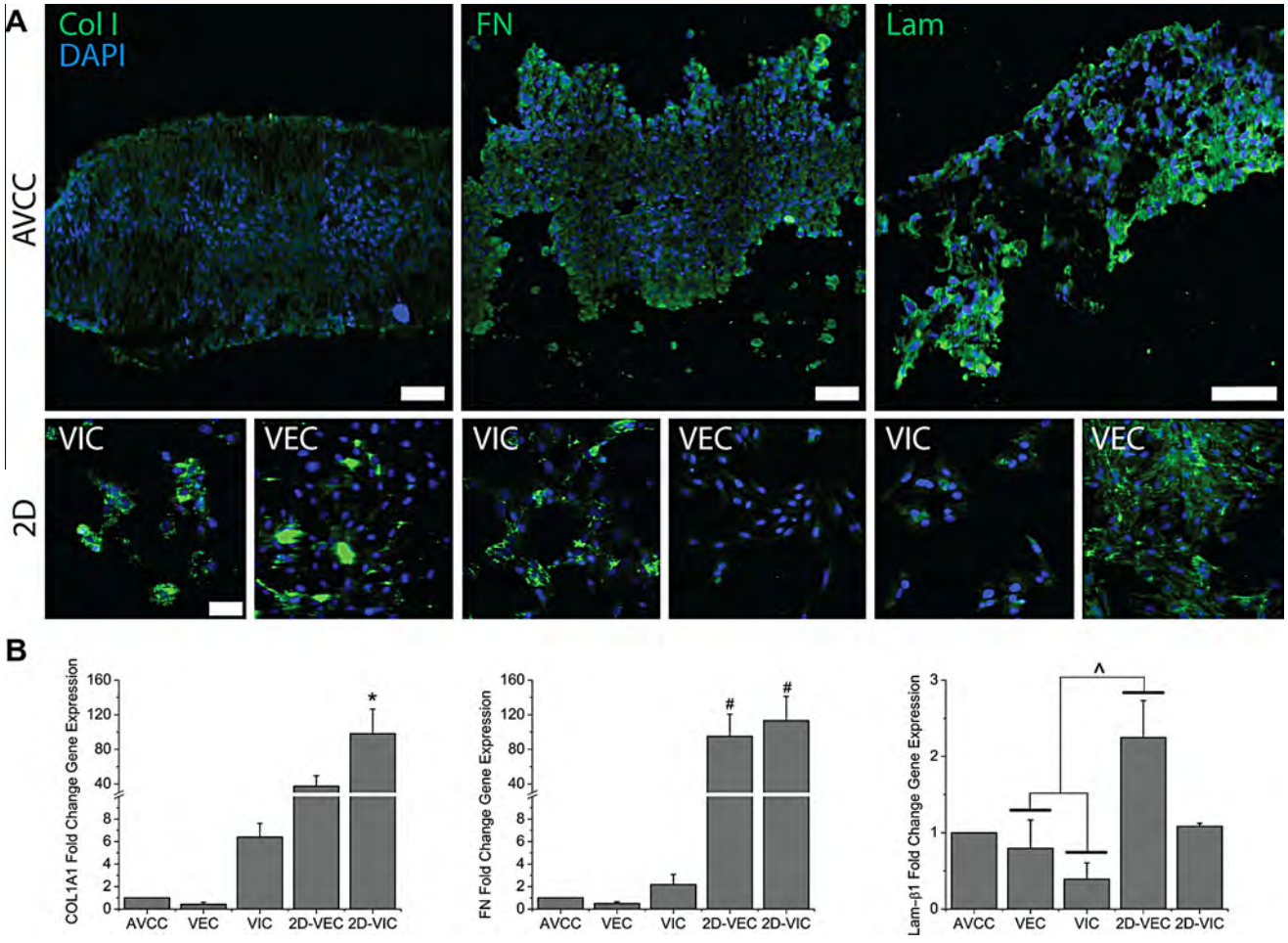

Fig. 5. (A) IHC stains (green) for the ECM components Col I, FN and Lam in the AVCCs and 2-D VIC and VEC cultures. Nuclei are counterstained using DAPI (blue). These ECM components are present and distributed throughout. In 2-D cultures, Col I and FN are more prominent with VICs, while Lam is more prominent with VECs. Scale bar = 50 μm. (B) qRT-PCR results for the ECM markers COL1A1, FN and Lam-β1. AVCC expressions of COL1A1 and FN were significantly less than those of 2-D VIC cultures. Lam-β1 for all groups was found to be statistically similar with expression in the AVCCs, but was significantly larger in 2-D VEC cultures, than 3-D monotype cultures. $^{*}P < 0.05$ vs. other groups; $^{\wedge}P < 0.05$ within bracket; $^{\#}P < 0.05$ vs. 3-D monotype cultures. Error bars represent standard error of the mean.

in engineered heart valves [7,59]. Further research using the AVCCs will require a deeper look into EnMT within the model, and could provide further understanding of EnMT and its utility.

An interesting result of this study was that 3-D VEC cultures showed significantly reduced expression of VWF, but increased expression of eNOS, relative to 2-D VEC cultures. As VWF and eNOS are both VEC-mediated proteins essential in facilitating endothelial pro- [60–62] and anti-thrombotic [63,64] responses, respectively, these results suggest the possibility that 3-D VEC cultures are in a more stable, anti-thrombotic state as opposed to 2-D cultures. The expression of these proteins is highly influenced by the surrounding environment. Previous studies have shown that when subjected to physiological shear flow conditions, vascular endothelial cells produce less pro-thrombogenic components such as VWF [65,66]. Thus, the changes in VWF and eNOS gene expression between two and three dimensions are possibly a result of the lower stiffness and higher cell density of the 3-D environment compared to the 2-D one. Therefore, culturing valvular cells in 3-D culture environments may not only maintain cell phenotype and function, but possibly enhance overall homeostatic cell function relative to 2-D cultures. Further studies of hemostatic factors within the AVCCs are necessary to understand its anti-thrombotic potential.

A major concern for this study was the choice of medium for co-culture, which was the medium used to culture VECs. In our other study using magnetic levitation to assemble co-cultures of the bronchiole, epithelial medium was used to maintain the co-cultures [41]. The rationale behind that choice was that epithelial cells were the definitive cell type of the bronchiole, and the most sensitive to serum due to fears of epithelial–mesenchymal trans-differentiation (EMT). As such, in that study, those co-cultures were cultured in epithelial medium with and without 1% FBS, and both cases showed the maintenance of epithelial phenotype and cell survival after 7 days [41]. Similarly, in this study VEC medium, which contained 2% FBS, was chosen over VIC medium, which contained 10% BGS, in order to preserve VEC phenotype against the possibility of EnMT. The results showed VEC phenotype was eventually maintained, as confirmed by positive stains and gene expression for CD31, VWF and eNOS, although co-localization with αSMA was present. Further research using this model should look at further optimization of the medium used for co-culture.

5. Conclusions

Magnetic levitation was successfully used to assemble co-cultures using VICs and VECs. Immunostaining and gene expression analysis were used to verify that the phenotype and functions of both cell types were maintained, and relevant ECM was formed. The result is a model that maintains phenotypes and induces ECM formation with a faster assembly time compared to other co-cultures. The AVCCs could in the future be used for a wide

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variety of experiments, such as those involving mechanobiology, or the progression of CAVD. This study also affirms the versatility and utility of magnetic levitation to create representative 3-D cell culture models.

Author disclosure statement

The University of Texas M.D. Anderson Cancer Center (UTMDACC) and Rice University, along with their researchers, have filed patents on the technology and intellectual property reported here. If licensing or commercialization occurs, the researchers are entitled to standard royalties. Glauco R. Souza, Robert M. Raphael and T.C. Killian have equity in Nano3D Biosciences, Inc. UTMDACC and Rice University manage the terms of these arrangements in accordance to their established institutional conflict-of-interest policies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.09.003.

Appendix B. Figures with essential colour discrimination

Certain figures in this article, particularly Figs 1–5, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2013.09.003.

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