Author version

Multilayer 3D Filter Paper Constructs for the Culture and Analysis of Aortic Valvular Interstitial Cells

Matthew C. Sapp, Hannelle J. Fares, Ana C. Estrada, K. Jane Grande-Allen

This manuscript was published in *Acta Biomaterialia*.

The publisher's version can be found at:

<http://www.sciencedirect.com/science/article/pii/S174270611400542X>

Cite as:

Sapp MC, Fares HJ, Estrada AC, Grande-Allen KJ. Multilayer three-dimensional filter paper constructs for the culture and analysis of aortic valvular interstitial cells. *Acta Biomater* 2015;13:199–206.

Multilayer 3D Filter Paper Constructs for the Culture and Analysis of Aortic Valvular Interstitial Cells

Matthew C. Sapp¹, Hannelle J. Fares¹, Ana C. Estrada¹, K. Jane Grande-Allen^{*,1}

Short title: Paper-Based Valvular Interstitial Cell Culture

*Address for correspondence:

K. Jane Grande-Allen, Ph.D.

Rice University

Dept of Bioengineering, MS 142

6100 Main St.

Houston, TX 77005

Phone: 713-348-3704

Fax: 713-348-5877

Email: grande@rice.edu

¹Department of Bioengineering, Rice University, Houston, TX, 77005

Abstract

Culturing aortic valvular interstitial cells in an environment that models the aortic valve is an essential step towards understanding the progression of calcific aortic valve disease. Here the adaption of a 3D stacked paper-based culture system is presented for analyzing valve cells in a thick collagen gel matrix. Filter paper layers, modeled after a 96-well plate design, were printed with a wax well-plate template and then seeded with valve cell and collagen mixtures that quickly gelled into 3D cultures. Stacking these layers permitted extensive customization of culture thickness and cell density profiles to model the full thickness of native valve tissue. Aortic valvular interstitial cells seeded into the paper-based constructs consistently demonstrated high survival up to 14 days of culture with significant increases in cell number through the first 3 days of culture. After 4 days following seeding, valve cells in single layer cultures showed reduced smooth muscle α -actin expression with a stabilized cell density, suggesting a transition from an activated phenotype to a more quiescent state. Valve cells in multilayer cultures demonstrated the ability to migrate from layer to layer and had the highest smooth muscle α -actin expression in areas with predicted low oxygen tensions. These results establish the filter paper-based method as a viable culture system for analyzing valve cells in an *in vitro* 3D model of the aortic valve.

Key Words

Cell activation

Collagen

ECM (extracellular matrix)

Heart valve

1. Introduction

Calcific aortic valve disease (CAVD) is the progressive hardening and calcification of the aortic valve in a process that is currently irreversible. With valve replacement as the only viable option, recent research has focused on more robust tissue valve replacements and noninvasive methods for treating or preventing valve disease [1,2]. Aortic valvular interstitial cells (VICs) have been targeted in studies on valve calcification because of their role in the production and turnover of leaflet extracellular matrix (ECM) [3,4]. Comprising the majority of the cells within the interior of the valve leaflets, VICs normally synthesize collagen, glycosaminoglycans, proteoglycans, and elastin to regulate the mechanics of the leaflet [5]. However, in diseased aortic valves, these cells have been shown to synthesize excessive amounts of collagen and create calcific nodules [6,7]. The phenotype of these cells are dependent on ECM remodeling as VICs are similar to fibroblasts when quiescent, and myofibroblasts when activated [8]. The role of VICs in the progression of CAVD continues to be widely debated, although VICs have been shown to transition to an osteoblast-like phenotype and create calcific nodules in culture [9]. It is important to note, however, that VICs do not become true osteoblasts but maintain unique distinguishing characteristics such as different expression levels of alkaline phosphatase and osteocalcin [10]. Altering matrix turnover and calcification potential in VICs has been a major goal in heart valve research. Furthermore, establishing the relationship between changes in VIC behavior and leaflet calcification remains a significant barrier to treating CAVD.

VICs have been studied extensively *in vitro* in 2D culture systems, and there has been an increasing focus on developing 3D culture systems that better model the *in vivo* leaflet environment. These studies have shown that VIC activation and calcification potential are measurably influenced by the substrate stiffness of the culture ECM or biomaterial [9]. Stiffer substrates such as polystyrene promote VIC calcification whereas collagen, hyaluronan, and

fibronectin gels or surface coatings reduce calcification [11–13]. Production of calcific nodules by VICs is a complicated process, however, involving multiple cell signaling pathways that are influenced by inflammatory factors, growth factors such as TGF- β , substrate stiffness, biological activity of the substrate, and oxygen and nutrient availability [12–17]. 2D culture systems, such as bioactive surface coatings on tissue culture polystyrene, have been used to successfully isolate many of these factors for analysis but yield limited results when used to replicate the complex array of factors involved in CAVD. Although some 3D systems have been used to modulate substrate stiffness, ECM composition, and various growth factors concurrently [18], 3D systems tend to be difficult to perform and are usually very time consuming. It is often difficult to mimic the same conditions between replicates and is challenging to control vital factors such as ECM composition and cell position when using these culture systems. Additionally, the aortic valve leaflet is composed of a complex tri-layered structure that varies in thickness by more than 1 mm throughout the cusp area [19,20]. Such significant changes in size of the leaflet's tri-layered structure are difficult to model with a 3D culture. Furthermore, there is a need for quantitative approaches to analyzing VIC behavior and expression throughout these 3D systems that is similar to the quantification capabilities of an ELISA. Creating a culture system that can incorporate such factors more effectively holds promise for identifying important components of the valve that initiate and sustain calcification.

One such system, the cells-in-gels-in-filter-paper system developed by the Whitesides group, offers the potential to culture VICs in a variety of highly-controlled 3D environments in an efficient and inexpensive manner [21]. Paper-based research systems have been a topic of great interest due to their cost-effectiveness, cell compatibility, non-toxic nature, and high-throughput capabilities. Originally designed to study breast cancer cells, the cells-in-gels-in-

filter-paper platform was created by printing and melting wax barriers onto sheets of highly porous filter paper. The sheets were then stacked to create 3D culture wells that were modeled after the spatial dimensions of a 96-well plate. The method avoids the difficulty of analyzing each 3D well separately by imaging each of the constructs with a gel scanner. Subsequent studies from the same research group have expanded the filter paper method to include a polyethylene (terephthalate) substrate and to have the ability to analyze the effects of soluble compounds on cell behavior [22,23]. An additional study used the system to study ischemia in cardiomyocytes [24].

In this work, the filter paper system has been adapted and enhanced for culturing VICs in thick collagen gel cultures with the ability to analyze viability and smooth muscle alpha actin (SM α A) expression. Specifically, a new type of gel has been added to the culture system and the imaging and analyzing capabilities have been improved. With these additions, the system is now able to analyze fluorescence from immunocytochemistry (ICC) using both a confocal microscope and a gel scanner. In these studies, VICs were cultured in single filter paper layers to optimize seeding parameters and culture durations. In multilayer environments, VICs were cultured in models of different aortic valve leaflet thicknesses to monitor cell migration and quantify SM α A expression. Together, these experiments demonstrate the potential of the filter paper system for culturing VICs in disease models of the aortic valve.

2. Materials and methods

2.1 Cell culture

Aortic valves were dissected from 6-month-old pig hearts obtained from a local commercial abattoir (Fisher Ham and Meat, Spring, TX). VICs were isolated from all 3 aortic

valve leaflets via collagenase digests following previously described methods [25] and were cultured in Dulbecco's Modified Eagle Medium (DMEM, low glucose at 1 g / L) containing Ham's F12 (Hyclone, Logan, UT), 10% bovine growth serum (Hyclone, Logan, UT), and 1% antibiotic / antimyotic (Mediatech, Manassas, VA). Cells were incubated at 37 °C in 5% CO₂. The culture medium was changed every 2-3 days, and cells were passaged at 80-90% confluence. Experiments were performed on cells from passages 2-3.

2.2 Fabrication of filter paper culture sheets

Creation of the stacked filter paper sheet system has been described previously [21,26]. In this work, the system and methods have been adapted and optimized for use in VIC cultures. Briefly, #114 filter paper sheets (Whatman, Pittsburgh, PA) with 190 µm thickness were cut into rectangles measuring 125 x 180 mm. A wax printer (ColorQube 8570, Xerox, Norwalk, CT) was used to print 4 filter paper wax templates onto the surface of each filter paper sheet. Sheets were cut and trimmed into individual 48-well sections and baked at 150 °C for 3 minutes. Once melting of the wax was complete, sheets were submerged in ultrapure water and sterilized at 121 °C for 60 minutes. Sheets were removed from the water and dried under sterile conditions. Finally, the sheets were heated at 100 °C for 2 minutes to restore the hydrophobicity of the wax. Following melting and sterilization, wells in the filter paper sheets measured 6 mm in diameter.

2.3 Fabrication of collagen gels

Collagen gels were prepared with type I rat tail collagen (BD Biosciences, San Jose, California) at a final concentration of 2 mg / ml in 0.02M acetic acid and DMEM (Mediatech, Manassas, VA) solution. The acetic collagen mixture was neutralized with 1M NaOH to initiate

gelation. After neutralization, collagen solutions were mixed with VICs at a concentration of 10 million cells / ml, unless otherwise noted, and were seeded into filter paper wells before gelation occurred. 10 million cells / ml was chosen as the optimal seeding concentration based on previous optimizations of the filter paper and prior uses of the concentration in previous 3D studies on VICs [8,27].

2.4 Construction of culture system

Filter paper sheets were cultured individually or as stacked constructs in 150 mm tissue culture dishes (Corning, Corning, NY). VIC / collagen mixtures were seeded into 36 wells on a 48-well filter paper sheet using a repeater pipette (Eppendorf, Hauppauge, NY). The remaining 12 wells functioned as collagen-only controls. Specific numbers of replicates varied by experiment and are specified in the figure captions. Following seeding, collagen gels were allowed to solidify for 5 minutes before culture media was added to the culture dish. For multilayer cultures, sheets were stacked with the corresponding wells aligned on top of a metal plate. Once all layers were in position, a second plate was bolted to the bottom plate to compress the layers together. This compression ensured that the layers were in contact so that cells would be able to migrate from one well to another between sheets (Fig. 4A). The top and bottom steel plates contained openings to allow nutrient and oxygen flow and were used in all multilayer experiments excluding 1 experiment (Fig. 5D). In that experiment, the bottom plate was completely solid and did not allow oxygen or media to pass through to the bottom of the culture stack. In all experiments, the culture media was changed every 2-3 days.

2.5 Optimization of single layer filter paper

VICs were seeded in single layer cultures in varying concentrations and over different culture periods to determine the final VIC number based on initial seeding conditions (Fig. 2). VIC numbers were used to create a predictable VIC behavior profile from which final VIC numbers could be related to initial seeding conditions. Seeding concentrations ranged from 1-10 million cells / ml with culture durations of 0-14 days.

VICs were cultured in different collagen concentrations to determine if higher collagen content gels retained more VICs (Fig. 3). Collagen gel recipes were adjusted to ensure that all factors excluding collagen content were held consistent between groups. VICs were also cultured with and without collagen gels to examine the role of filter paper fibers in VIC retention within wells. For non-collagen conditions, VICs were mixed with media and seeded directly into wells. In all conditions, VIC / gel and VIC / media solutions were left in wells for 5 minutes before adding media to the culture system. Collagen gel concentrations ranged from 1-3 mg / ml and culture durations were from 0 to 10 days.

2.6 VIC migration in multilayer cultures

VICs were seeded at 10 million cells / ml into the central layer of a multilayer filter paper stack to examine VIC migration (Fig. 4). The remaining layers were seeded with collagen only. Experiments were conducted with different numbers of layers and varying culture durations to demonstrate the migration potential of VICs related to culture time and culture well thickness. Stacks ranged in size from 3-5 layers (0.6-1.0 mm) cultured over 3-7 days.

2.7 Investigation of VIC activation

VICs were seeded into a 7-layer stack, 5-layer stack, 3-layer stack, and a single layer piece of filter paper for comparison of VIC activation (Fig. 5 and Supplemental Fig. 1). VICs were seeded at 10 million cells / ml in all 7 layers of a large stacked culture with a closed bottom plate (Fig. 5D). For comparison, a 7-layer stack with open ends was seeded with VICs in all layers (Fig. 5C). A normal 5-layer stack and a 3-layer stack with open steel plates on both ends were also seeded with VICs in all layers. The stacked culture systems were used to determine VIC activation based on each layer at a single time point. The single layer system examined VIC activation over 4 days of culture.

2.8 Analysis of cell morphology, viability, and activation

The behavior of VICs in filter paper was examined by observing VIC number over time with varying cell densities in single and multi-layer environments. At the conclusion of each experiment, cells were incubated with 4 μ M of the vital dye calcein (Molecular Probes, Grand Island, NY) in media for 1 hour at 37 °C. Cells were then washed with phosphate buffered saline (PBS) and fixed using 4% paraformaldehyde prior to fluorescence analysis or confocal imaging (below).

Studies in both single and multilayer environments focused on changes in VIC activation over time with specific attention to seeding location. In select experiments, VIC activation was demonstrated with the commonly used VIC marker SM α A. In this process, ICC was performed on each culture using a mouse anti-SM α A primary antibody (Molecular Probes, Grand Island, NY) with incubation for 24 hours at 4 °C. The cultures were then incubated for 24 hours at 4 °C in a goat anti-mouse Alexa Fluor 555 secondary antibody. Each sheet of paper was washed with

0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for 15 minutes and blocked with 10% goat serum in PBS for 1 hour prior to antibody labeling. Antibody solutions were pipetted directly into each culture well.

Fig. 1. (A) Sheets of 0.2 mm thick filter paper were printed with a well-plate mask by a wax solid-ink cartridge printer. The wax was melted through the thickness of the paper and the paper was sterilized at 121 °C. Cell / gel mixtures were seeded by a repeat pipette into each well. For single layer 3D cultures, individual sheets were cultured in a 150 mm tissue culture dish. For multilayer cultures, sheets were stacked with corresponding wells aligned and were compressed together using steel plates. Following culture, cells were identified with immunocytochemistry or

viability assays and imaged with a gel scanner or confocal microscope. Here, fluorescence in each well is the result of calcein staining. (B) Filter paper fibers and VIC nuclei were visualized with DAPI (left). Viable VICs were identified with calcein staining (right). Scale bar = 100 μm .

A gel imager (Gel Loic 4000 Pro, Carestream Health, Rochester, NY) was used to image the total fluorescence from each well. Following fixation and ICC, filter paper sheets were separated and arranged in the gel scanner window to allow for simultaneous imaging of all experimental samples. Images were output as a 16 bit grayscale *.tif file that was analyzed using a custom script in Matlab (Mathworks, Natick, MA) (Fig. 1A). The original script was generously provided by the Whitesides lab [21] and was modified for analyzing ICC in VIC cultures. Final values for each sample reflect the average fluorescence of that well. Wells of the same set were averaged together to produce a final numerical value. In single layer experiments, all values were normalized to the highest recorded value for that experiment and to the background recorded in collagen-only wells. In multilayer experiments, all values were normalized to the highest and lowest values recorded by the gel scanner.

2.9 Confocal microscopy imaging

Samples were prepared for confocal imaging by removing several randomly chosen wells after calcein staining or ICC processing and mounting them onto a glass slide with a glass coverslip and Vectashield Mounting Media (Vector Laboratories, Burlingame, CA). DAPI nuclear stain was added to selected wells via the mounting media. A Nikon A1-Rsi confocal microscope (Nikon, Melville, NY) was used to capture stacked images traversing the entire thickness of a single filter paper well (190 μm) within one sheet of filter paper.

2.10 Statistical analysis

Experiments were often performed 2-3 times and representative data is presented as the mean plus or minus standard error of the mean from one experiment. All experiments other than those in Fig. 3B were analyzed for significant differences using an analysis of variance (ANOVA) test with a post hoc Tukey's test. The Tukey's test was performed using JMP Pro 10 (SAS Institute Inc., Cary, NC). Significance in Fig. 3B was determined using a student's t-test. Groups were considered significant when $p < 0.05$. Sample sizes ranged from 31-36 per group as indicated by figure legends. Data was reported as the mean normalized fluorescence with standard error. Letters on figures indicate significant differences. Bars in the bar graphs that share the same letter are not significantly different from one another. Bars that contain different letters are significantly different from one another. In single layer experiments, these letters represent significant differences in VIC number or SM α A expression per cell in regard to time, seeding concentration, or collagen concentration. In multilayer experiments, these letters represent significant differences in VIC numbers per layer or SM α A expression per layer in regard to time or number of layers.

Fig. 2. VIC number was studied in single layer sheets. Letters on each bar represent significant differences in cell number between initial seeding concentrations (Fig. 2A-B) and culture durations (Fig. 2C-D). Bars that share the same letter are not significantly different. Bars with different letters are significantly different from one another. Initial VIC seeding concentration was 10 million cells / ml (Fig. 2C-D). Significance was determined using an ANOVA with a post-hoc Tukey's test. The dashed line represents initial cell seeding concentration (Fig. 2C-D).

(A) Calcein fluorescence was proportional to the number of VICs seeded into a well (n=36). (B) Wells initially seeded with more VICs continued to have a greater number of VICs at 3 days (n=36). (C) VIC number in each well slightly increased over 10 hours following seeding (n=31). (D) VIC number per well stabilized within 3 days following seeding, suggesting a slowing in proliferation (n=36).

3. Results

3.1 Analyzing the filter paper construct

Printing and melting wax throughout the thickness of each sheet successfully allowed for the formation of stackable culture wells. In the gel scanner images, higher fluorescence appeared as white areas and absence of fluorescence was black (Fig. 1A). Confocal z-stacked images of filter paper wells demonstrate the position and cell morphology of the VICs following seeding and at 14 days (Fig. 1B). DAPI stained the cell nuclei, as well as the filter paper fibers. Calcein stained only the cells and did not produce any noticeable background signal. Rated for 25 μm particle retention, the cellulose filter paper fibers were generally 30-50 μm in diameter with approximately 50-500 μm spacing between fibers. VICs appeared rounded and evenly distributed immediately following seeding, with paper fibers causing occasional separation between groups of VICs. At 14 days, VIC morphology was more elongated and VICs were more widely spaced from one another (Fig. 1B).

3.2 Changes in VIC density over time

VIC number given by calcein fluorescence was found to follow a near-linear relationship with initial seeding density (Fig. 2A). This relationship continued after 3 days of culture. Two

seeding densities were altered to demonstrate that the near-linear relationship remained present for additional seeding densities between 1 and 10 million cells / ml (Fig. 2B). VIC number slightly increased over 10 hours following seeding, indicating high VIC survival after an initial loss of cells during seeding (Fig. 2C). VIC number continued to increase through 3 days following seeding (Fig. 2D). After 3 days, VIC number stabilized with calcein fluorescence remaining constant.

VIC number was found to be related to the collagen composition of the collagen gels (Fig. 3A). VICs seeded in gels with a low collagen concentration (1 mg / ml) retained the least number of VICs within the filter paper wells. Gels with higher collagen concentrations (2 or 3 mg / ml) retained significantly more VICs. Although VIC number was highest in the 3 mg / ml concentration, 2 mg / ml was chosen as the standard collagen concentration for all other experiments. Since 3 mg / ml collagen gels generally have less than 5% of their total volume available for customization as a result of the concentration of the collagen stock solution, 2 mg / ml collagen gels provide much greater flexibility when adding other solutions to the gels. Future studies with the filter paper system may be conducted with additional ECM components in the collagen gels, requiring a greater volume for customization that is found in the 2 mg / ml collagen gels. As a control to the collagen gels, VICs were seeded in filter paper wells without the encapsulating collagen gel. VIC retention and survival in these wells was poor and a significant drop in cell number was evidenced by decreased fluorescence after 10 days in culture (Fig. 3B). The cells remaining in the media-only wells at the 10 day timepoint were spherical in shape in contrast to the normal, elongated VIC morphology apparent within collagen gels (Fig. 3C).

3.3 VIC migration

Multilayer filter paper scaffolds expanded the thickness of single layer 3D culture wells by adding layers with thicknesses of approximately 200 μm . VICs were seeded into the middle layer of 3- and 5-layer cultures (0.6 mm and 1.0 mm thick respectively) to determine the extent of VIC migration throughout the layers over time (Fig. 4A). These thicknesses were chosen because they encompass the range of thicknesses of the basal region (0.69 to 0.86 mm) of an adult valve leaflet [28]. In both 3- and 5-layer culture conditions, VICs were able to migrate to all layers within 3 days (Fig. 4B, Fig. 4D), demonstrating the capability to move through a scaffold as thick as 1 mm. VICs remained in high cell densities throughout all layers when assayed at day 5 (Fig. 4C). Layer compression was also inspected as a variable in VIC migration behavior using a 5-layer culture setup that was not as tightly compressed as the normal migration cultures (Fig. 4E). This setup showed that VICs were still able to migrate from layer to layer and exhibit a similar distribution profile without a strong reliance on the amount of compression on the scaffolds.

3.4 SM α A expression in VICs

Expression of SM α A is often used as an indicator of VIC activation and can be employed to show the potential progression of VICs towards a calcific phenotype [29]. VIC expression of SM α A was shown for filter paper culture wells (Fig. 5A). Changes in VIC SM α A expression in single- and stacked-layer cultures were studied. SM α A expression per cell over a period of 4 days was examined in single layers (Fig. 5B). By 4 days, SM α A expression per cell dropped to levels lower than those during initial cell seeding by 30%.

Stacked cultures were compressed by either 2 open plates, or 1 open plate (top of stack) and 1 solid plate (bottom of stack). A 7-layer culture with 2 open plates was cultured for 5 days (Fig. 5C). SM α A expression per cell was greatest in the center of the stacked culture. A 7-layer culture with an open plate above layer 1 and closed plate below layer 7 was also cultured for 5 days (Fig. 5D). In this arrangement, VICs positioned farther from the open top plate exhibited decreased survival with an increase in SM α A levels. SM α A expression per cell was greatest in the bottom 3 layers of the culture stack. 3- and 5-layer stacked cultures were attempted but only showed slight differences in SM α A expression per cell from layer to layer. These figures have been included in the supplementary material for completeness. SM α A expression per cell was lowest in the middle layer of the 3-layer stack (Supplemental Fig. 1A). Similar to the 3-layer culture, VIC SM α A expression per cell in the 5-layer culture was highest in the first and last layers and lowest in the middle layer (Supplemental Fig. 1B).

Fig. 3. (A) Collagen I concentration in collagen gels impacted overall VIC number at 3 days culture time. Letters represent significant differences. Bars that share the same letter are not

significantly different. Bars with different letters are significantly different from one another. Higher concentrations resulted in greater VIC number (n=33). (B) Few VICs remained in gel-less wells as demonstrated by a significant decrease in cell number over 10 days (n=33). Stars represent significant differences. In comparison, 60% of the original VIC seeding number was present in 2 mg / ml collagen gels at 10 days (n = 36). (C) Seeding VICs directly into filter paper without gels versus the use of collagen gels. Most VICs that were seeded without collagen were not present at 10 days. VICs seeded with collagen gels remained in the wells in much higher numbers and migrated throughout. Scale bar = 100 μ m.

4. Discussion

The stacked filter paper method is a promising VIC culture system that allows customization of ECM combined with the ability to stack individual layers for a controllable thickness of the total culture. Seeding VICs into layers before the experiment and unstacking those layers after culture circumvents time-consuming sectioning of the 3D culture and slide-by-slide histological analysis. Furthermore, destacking layers and imaging with a gel scanner minimizes damage to the culture that may occur with more destructive analysis techniques. In addition, analysis of all of the layers with a gel scanner eliminates the need for imaging with a slide scanner or microscope and dramatically reduces time of analysis and imaging. However, wells can still be effectively imaged with a confocal microscope if desired (Fig. 1B and Fig. 5A). Recent updates to the culture system have also established light and fluorescent microscopy with an inverted light microscope as an acceptable imaging method for polymer-based cultures [22].

A

Initial Seeding Configuration

B

3 Day Migration

C

5 Day Migration

D

3 Day Migration

E

Loosely Compressed

Fig. 4. (A) VICs were seeded into the middle layer of either a 3-layer or 5-layer filter paper construct. The remaining 2 or 4 layers were seeded with collagen only. Metal plates were used to compress the layers together to facilitate migration from layer to layer. (B) VICs were able to migrate through 2 layers (0.4 mm total) in each direction from the center layer within 3 days. Cells were evenly distributed in the middle 3 layers with a lower number of VICs in the first and

fifth layers (n=36). The yellow arrow represents the original VIC seeding layer. Letters on each bar represent significant differences of VIC number among layers. Bars that share the same letter are not significantly different. Bars with different letters are significantly different from one another. (C) A similar VIC distribution profile to (A) was observed with the highest number in VICs in the middle 3 layers with a maximum of 25% fewer cells in the fifth layer at 5 days culture time (n=36). (D) VICs also migrated throughout a 3-layer culture within 3 days culture time (n=36). (E) VICs were able to migrate throughout a loosely compressed culture stack within 7 days, indicating that high compression of the stacked paper by metal plates was not necessary for VIC migration from layer to layer (n=36). Loosely compressed is defined as a stacked culture that was confined between two steel plates that were not tightly screwed together.

In this study, VICs maintained a high cell density in the stacked paper culture system with culture durations ranging from initial seeding to long-term culture up to 2 weeks. A previous study with VICs in thick collagen gels also showed high survival over time [30]. VICs can be seeded into each layer with highly controlled parameters, such as cell concentration, and the VICs exhibit predictable and consistent cell densities over time (Fig. 2). VICs also produced reliable results in large multi-layered cultures that allowed VICs to migrate from layer to layer. These migration data showed that each stacked well functioned as a single culture (Fig. 4). VICs were able to move freely from layer to layer and occupied each layer in relatively equal proportions, provided that adequate oxygen and nutrients were available. Interestingly, VIC migration continued to occur when layers were not tightly compressed, suggesting that VICs may be able to migrate short distances through the media. Future studies with the filter paper system will utilize degradable hydrogels to better control migration.

A Single Layer Cultures

C Multilayer Cultures

7 Layer - Open End

B Single Layer over Time

D 7 Layer - Blocked End

Fig. 5. SMαA expression in VIC filter paper cultures. (A) Top – Calcein fluorescence (green) and SMαA expression (red) in a single layer filter paper collagen gel VIC culture. Bottom – DAPI image (blue) and 3-channel overlay of the VIC culture. VICs in the filter paper demonstrated migration, cell elongation, and SMαA expression. Scale bar = 50 μm. (B) In single layer cultures, SMαA expression per cell initially increased and then significantly declined by 30% (compared to day 0) at 4 days of culture (n = 36). Letters on each bar represent significant differences of VIC SMαA per cell between days. Bars that share the same letter are not

significantly different. Bars with different letters are significantly different from one another. (C) SM α A expression per cell in a 7-layer culture with open ends. As VIC number decreased toward the middle of the culture stack, SM α A expression per cell increased. Culture duration was 5 days (n = 36). Letters on each bar represent significant differences of VIC SM α A per cell between layers. (D) SM α A expression per cell in a 7-layer culture with a blocked bottom end metal plate. As VIC number decreased toward the bottom of the culture stack, SM α A expression per cell increased. Culture duration was 5 days (n = 31).

One of the major indicators used to represent altered VIC behavior and activation has been SM α A expression. While positive SM α A expression may not always be necessary for VICs to transition to an osteoblast-like phenotype [10], it is often used to identify VICs undergoing calcification and is found in much higher amounts in diseased aortic valves [31]. Therefore, identification of this marker in 3D VIC cultures is essential. Within the 3D filter paper culture system, VICs showed higher SM α A expression at the bottom of a stacked culture with a blocked end. In a stacked culture with both ends open, SM α A expression was highest in the center and lowest near the openings. In these models of ischemia, SM α A expression increased in areas of presumably greater hypoxic stress. Future studies will examine these phenomena further to better understand the role of oxygen in SM α A expression. In single layers, SM α A expression was much lower by 4 days, indicating a VIC transition from an activated state to a more quiescent state that is often found in healthy valves *in vivo*. Reduction in VIC SM α A expression likely occurred due to culture on collagen-I substrates, as shown by collagen's ability to reduce VIC calcification found in earlier studies with collagen [11].

There are several notable limitations for the filter paper culture system including difficulty in tensile straining the cultures. However, standard compression and hydrostatic compression are viable alternatives and will be explored in future studies. Although cell position can be determined on a layer to layer basis during seeding, cell position within each layer cannot be controlled. Thinner sheets of filter paper can be used to more accurately position cells within a large 3D paper construct if desired. Additionally, very thin sheets of filter paper can be positioned between layers to confine cell movement within single layers. The paper fibers also resist contraction of the gels, although inhibition of contraction does allow VIC cultures to have defined dimensions before and after experiments. Furthermore, contraction of collagen gel cultures often prevents the use of specific collagen gel concentrations. With the filter paper system, VIC cultures retained their original dimensions with high collagen content up to 3 mg / ml (Fig. 3A). Finally, there could be the concern that the filter paper fibers disrupt VIC networks while providing a stiff attachment substrate that affects VIC activation. Here it was shown that few VICs remain in culture in the absence of ECM (Figs. 3B-C). This suggests that VICs do not preferentially attach to filter paper fibers. Additionally, confocal stacked images show that VIC SM α A expression does not change with regard to the proximity of filter paper fibers (Fig. 5A).

With these limitations considered, the stacked filter paper system is a viable culture construct for modeling VIC calcification in the aortic valve. This system contains a variety of advantages over commonly used 2D and 3D culture systems and holds promise for improving the current state of knowledge of the role of VICs in CAVD. Future studies will aim to promote calcification in these 3D cultures and study changes in VIC behavior. Furthermore, future studies will utilize the multilayer system to incorporate multiple types of ECM to better model the aortic valve structure and study the impact of ischemia on VICs. An example of this setup would

include adding hyaluronan to layers in the center and using collagen for the outer layers.

Additional cell types such as valvular endothelial cells (VECs) could also be added to the outer layers to study VIC / VEC interactions.

5. Conclusions

The filter paper-based system presented here demonstrates a novel form of culturing VICs in a method that quantifies ICC fluorescence while simplifying the creation and analysis of complex 3D culture systems. Adapted from a system used to culture cancer cells [21], the filter paper culture platform creates new opportunities for studying VICs in both normal and diseased models of the aortic valve. In this study, VIC behavior in the 3D filter paper system was imaged and quantified using a confocal microscope and a gel scanner. When seeded with collagen, VICs maintained a high cell density in all culture constructs with a diminishing SM α A expression over time in single layers. These results indicate that the filter paper-based culture system is an effective platform for studying VIC expression in environments with customizable thicknesses.

Acknowledgements

This work was funded by the AHA Innovative Research Grant number 11IRG5550052. The authors would like to thank the Farach-Carson Lab for generously providing the fluorescent gel scanner, the Whitesides Group at Harvard University for the cell quantification Matlab code, Dr. Ratmir Derda for assistance in synthesizing the filter paper system, and Eli Mondragon for optimizing the filter paper wells. The authors would also to thank Anna Handelman and Henry Deng for their help with the ANOVA statistics.

References

- [1] Tseng H, Cuchiara ML, Durst C, Cuchiara MP, Lin CJ, West JL. Fabrication and mechanical evaluation of anatomically-inspired quasilaminate hydrogel structures with layer-specific formulations. *Ann Biomed Eng* 2013;41:398–407.
- [2] Benton J, Kern HB, Leinwand L, Mariner PD, Anseth KS. Statins block calcific nodule formation of valvular interstitial cells by inhibiting alpha-smooth muscle actin expression. *Arterioscler Thromb Vasc Biol* 2009;29:1950–7.
- [3] Aikawa E, Whittaker P, Farber M, Mendelson K, Padera RF, Aikawa M, Schoen FJ. Human semilunar cardiac valve remodeling by activated cells from fetus to adult: implications for postnatal adaptation, pathology, and tissue engineering. *Circulation* 2006;113:1344-52.
- [4] Dreger S, Thomas P, Sachlos E, Chester AH, Czernuszka JT, Taylor PM, Yacoub MH. Potential for synthesis and degradation of extracellular matrix proteins by valve interstitial cells seeded onto collagen scaffolds. *Tissue Eng* 2006;12:2533–40.
- [5] Masters KS, Shah DN, Walker G, Leinwand L, Anseth KS. Designing scaffolds for valvular interstitial cells: cell adhesion and function on naturally derived materials. *J Biomed Mater Res A* 2004;71:172–80.
- [6] Hinton RB, Lincoln J, Deutsch GH, Osinska H, Manning PB, Benson DW, Yutzey KE. Extracellular matrix remodeling and organization in developing and diseased aortic valves. *Circ Res* 2006;98:1431–8.
- [7] Kumar A, Wiltz DC, Grande-Allen KJ. Gentamicin reduces calcific nodule formation by aortic valve interstitial cells in vitro. *Cardiovasc Eng Technol* 2012;4:16-25.

- [8] Benton J, DeForest CA, Vivekanandan V, Anseth KS. Photocrosslinking of gelatin macromers to synthesize porous hydrogels that promote valvular interstitial cell function. *Tissue Eng Part A* 2009;15:3221-30.
- [9] Yip CYY, Chen J-H, Zhao R, Simmons C. Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix. *Arterioscler Thromb Vasc Biol* 2009;29:936-42.
- [10] Monzack EL, Masters KS. Can valvular interstitial cells become true osteoblasts? A side-by-side comparison. *J Heart Valve Dis* 2011;20:449-63.
- [11] Rodriguez KJ, Masters KS. Regulation of valvular interstitial cell calcification by components of the extracellular matrix. *J Biomed Mater Res A* 2009;90:1043-53.
- [12] Rodriguez KJ, Piechura LM, Masters KS. Regulation of valvular interstitial cell phenotype and function by hyaluronic acid in 2-D and 3-D culture environments. *Matrix Biol* 2011;30:70-82.
- [13] Benton JA, Kern HB, Anseth KS. Substrate properties influence calcification in valvular interstitial cell culture. *J Heart Valve Dis* 2008;17:689-99.
- [14] Wang H, Haeger SM, Kloxin AM, Leinwand LA, Anseth KS. Redirecting valvular myofibroblasts into dormant fibroblasts through light-mediated reduction in substrate modulus. *PLoS One* 2012;7:e39969.
- [15] Walker G, Masters KS, Shah DN, Anseth KS, Leinwand L. Valvular myofibroblast activation by transforming growth factor-beta: implications for pathological extracellular matrix remodeling in heart valve disease. *Circ Res* 2004;95:253-60.
- [16] Gu X, Masters KS. Regulation of valvular interstitial cell calcification by adhesive peptide sequences. *J Biomed Mater Res A* 2010;93:1620-30.

- [17] Cushing MC, Liao J-T, Anseth KS. Activation of valvular interstitial cells is mediated by transforming growth factor-beta1 interactions with matrix molecules. *Matrix Biol* 2005;24:428–37.
- [18] Benton JA, Fairbanks BD, Anseth KS. Characterization of valvular interstitial cell function in three dimensional matrix metalloproteinase degradable PEG hydrogels. *Biomaterials* 2009;30:6593–603.
- [19] Stephens EH, Grande-Allen KJ. Age-related changes in collagen synthesis and turnover in porcine heart valves. *J Heart Valve Dis* 2007;16:672–82.
- [20] Weind KL, Boughner DR, Rigutto L, Ellis CG. Oxygen diffusion and consumption of aortic valve cusps. *Am J Physiol Heart Circ Physiol* 2001;281:H2604–11.
- [21] Derda R et al. Multizone Paper Platform for 3D Cell Cultures. *PLoS One* 2011;6:e18940.
- [22] Simon K, Park KM, Mosadegh B, Subramaniam AB, Mazzeo AD, Ngo PM, Whitesides GM. Polymer-based mesh as supports for multi-layered 3D cell culture and assays. *Biomaterials* 2014;35:259–68.
- [23] Deiss F, Mazzeo A, Hong E, Ingber DE, Derda R, Whitesides GM. Platform for high-throughput testing of the effect of soluble compounds on 3D cell cultures. *Anal Chem* 2013;85:8085–94.
- [24] Mosadegh B, Dabiri BE, Lockett MR, Derda R, Campbell P, Parker KK, Whitesides GM. Three-Dimensional Paper-Based Model for Cardiac Ischemia. *Adv Healthc Mater* 2014;3:1036-43.
- [25] Stephens EH, Carroll JL, Grande-Allen KJ. The use of collagenase III for the isolation of porcine aortic valvular interstitial cells: rationale and optimization. *J Heart Valve Dis* 2007;16:175–83.

- [26] Derda R, Laromaine A, Mammoto A, Tang SKY, Mammoto T, Ingber DE, Whitesides GM. Paper-supported 3D cell culture for tissue-based bioassays. *Proc Natl Acad Sci U S A* 2009;106:18457–62.
- [27] Duan B, Hockaday LA, Kang KH, Butcher JT. 3D Bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels. *J Biomed Mater Res A* 2012:1–10.
- [28] Weind KL, Ellis CG, Boughner DR. Aortic valve cusp vessel density: Relationship with tissue thickness. *J Thorac Cardiovasc Surg* 2002;123:333–40.
- [29] Wyss K, Yip CYY, Mirzaei Z, Jin X, Chen J-H, Simmons CA. The elastic properties of valve interstitial cells undergoing pathological differentiation. *J Biomech* 2012;45:882–7.
- [30] Gould RA, Chin K, Santisakultarm TP, Dropkin A, Richards JM, Schaffer CB, Butcher JT. Cyclic strain anisotropy regulates valvular interstitial cell phenotype and tissue remodeling in three-dimensional culture. *Acta Biomater* 2012;8:1710–9.
- [31] Wirrig EE, Hinton RB, Yutzey KE. Differential expression of cartilage and bone-related proteins in pediatric and adult diseased aortic valves. *J Mol Cell Cardiol* 2011;50:561–9.

Supplementary Material

Supplemental Fig. 1. SMαA expression per cell in 3- and 5-layer culture stacks with open ends. Bars that share the same letter are not significantly different. Bars with different letters are significantly different from one another. (A) SMαA expression per cell in a 3-layer migration culture (n = 32). SMαA expression was present across all layers and lowest in the center layer. (B) SMαA expression per cell in a 5-layer culture. SMαA continued to be expressed by VICs across all layers with highest expression in layers 1 and 5 and lowest expression in the middle of the culture stack (n=36). Levels of SMαA expression / cell in 3- and 5-layer cultures occurred in different expression profiles compared to the 7-layer cultures (Fig. 5C-D). Specifically, 3- and 5-layer cultures had the highest SMαA levels at the outer layers of the cultures whereas 7-layer cultures had high SMαA expression in areas furthest away from the oxygen and nutrient source. It is hypothesized that 3- and 5-layer cultures are not large enough to be significantly affected by differences in oxygen levels whereas 7-layer cultures experience a change in SMαA expression due to low oxygen levels.