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Microarray analyses to quantify advantages of 2D and 3D hydrogel culture systems in maintaining the native valvular interstitial cell phenotype

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Abstract

Valvular interstitial cells (VICs) actively maintain and repair heart valve tissue; however, persistent activation of VICs to a myofibroblast phenotype can lead to aortic stenosis. To better understand and quantify how microenvironmental cues influence VIC phenotype and myofibroblast activation, we compared expression profiles of VICs cultured on poly(ethylene glycol) (PEG) gels to those cultured on tissue culture polystyrene (TCPS), as well as fresh isolates. In general, VICs cultured in hydrogel matrices had lower levels of activation (<10%), similar to levels seen in healthy valve tissue, while VICs cultured on TCPS were ~75% activated myofibroblasts. VICs cultured on TCPS also exhibited a higher magnitude of perturbations in gene expression than soft hydrogel cultures when compared to the native phenotype. Using peptide-modified PEG gels, VICs were seeded on (2D), as well as encapsulated in (3D), matrices of the same composition and modulus. Despite similar levels of activation, VICs cultured in 2D had distinct variations in transcriptional profiles compared to those in 3D hydrogels. Genes related to cell structure and motility were particularly affected by the dimensionality of the culture platform, with higher expression levels in 2D than in 3D. These results indicate that dimensionality may play a significant role in dictating cell phenotype (e.g., through differences in polarity, diffusion of soluble signals), and emphasize the importance of using multiple metrics when characterizing cell phenotype.

Keywords

heart valve; valvular interstitial cells; hydrogel; three-dimensional cell culture; ECM; microarray

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1. Introduction

VICs are the primary cell type found within heart valves. In patients with aortic stenosis, VICs remodel their surrounding extracellular matrix (ECM) in a manner that causes pathological stiffening of the valve [1,2]; this fibrotic stiffening can then lead to regurgitation or obstruction of blood flow [3]. Currently, there are no pharmaceutical therapeutics that have proven to be effective for the reversal of aortic stenosis, and the main treatment option is surgical replacement of the valve [4]. While advances have occurred in minimally invasive valve replacement therapies, a better understanding of VIC biology may provide alternative solutions to valve replacement by focusing on reversal or slowing of disease progression. To address this need, *in vivo* models are highly relevant, but their complex nature makes it difficult to elucidate specific mechanisms of VIC activation and disease progression. In contrast, *in vitro* systems provide a high level of control, but these systems are limited by physiological relevance and must be evaluated for their ability to recapitulate mechanism of interest. For these reasons, *in vivo* and *in vitro* experiments are complementary and both approaches are needed.

In healthy cardiac valves, the majority of VICs exhibit a quiescent fibroblast phenotype [5]; however, in patients with valve disease, many VICs become activated to a myofibroblast phenotype. The VIC myofibroblast phenotype is characterized by the presence of prominent α-smooth muscle actin (αSMA) stress fibers and associated with increased proliferation, ECM remodeling, and cytokine secretion [2,6]. *In vitro* culture systems afford an opportunity to study this transition, especially as a function of VIC-matrix interactions and in the absence of the complex signaling milieu that occurs *in vivo*. However, dramatic changes occur in the VIC phenotype when they are isolated from valve tissue and cultured using traditional methods, and this aphysiological response can complicate the identification of new approaches to regulate the pathological VIC myofibroblast phenotype.

Culturing VICs on supra-physiologically stiff, 2D tissue culture polystyrene (TCPS) (i.e., > 5 orders of magnitude stiffer than compliant valves [7]) alters many of the signals that the cells receive [8]. Unfortunately, with VICs, some of the functions most highly correlated to valve disease progression are also the ones that are most dramatically affected by culture on TCPS, making it difficult to study this transition and/or its reversal. Specifically, plating VICs on TCPS leads to high levels of myofibroblast activation and renders it nearly impossible to study the quiescent fibroblast phenotype [8].

Wang *et al.* provided one of the first reports quantifying the effect of TCPS culture on porcine VICs by performing a microarray experiment to measure mRNA levels in freshly isolated VICs compared to VICs cultured on TCPS. Results showed that over 4000 genes were differentially regulated, which was two orders of magnitude higher than that observed with transforming growth factor-β (TGF-β) treatment [8]. This is significant, as TGF-β is a potent cytokine that is known to cause activation of myofibroblasts in many tissues, and mis-regulation of TGF-β signaling has been implicated in heart valve problems related to the use of the anti-obesity drug, FEN-PHEN [9]. Thus, microenvironment is hypothesized to play an important role in regulating the VIC phenotype, and Wang *et al.* further

demonstrated that culturing VICs on soft hydrogel substrates restored expression levels of many critical genes to levels measured in freshly isolated cells.

While seeding VICs on softer, more biomechanically relevant substrates instead of TCPS helps recapitulate some aspects of the native VIC phenotype, there are many differences between TCPS and hydrogel matrices, as well as many differences between the cellular microenvironment *in vivo* and hydrogel substrates *in vitro*. To begin to deconvolute some of these differences, this work aims to elucidate the effects of dimensionality of the matrix on VIC interactions and phenotype. Clearly, removing cells from their three-dimensional native environment and seeding them on two-dimensional surfaces can significantly affect their phenotype. As one specific example, previous reports have shown that increasing substrate modulus in 2D leads to higher levels of VIC myofibroblast activation [10], while the opposite effect was observed in 3D [11].

To evaluate the impact of microenvironment on VIC phenotype, additional metrics are necessary. αSMA stress fibers are a hallmark of the myofibroblast phenotype, but a more complete description is warranted, especially at the molecular level to better define the differences between VIC fibroblasts and myofibroblast. Such a global characterization would provide metrics to distinguish between populations that have similar levels of VIC activation, but potentially different functional characteristics. For example, nearly 100% of VICs cultured on TCPS either with or without TGF-β exhibit αSMA stress fibers; however, the addition of TGF-β does result in an increase in contractility and inhibition of proliferation and apoptosis [12]. These functional differences in VIC populations with equivalent activation levels demonstrate the importance of performing a more in-depth, systematic characterization of VIC phenotype rather than relying on a single metric. We hypothesize that populations of VICs cultured in different environments that have the same percentage of activated myofibroblasts can exhibit very distinct transcriptional profiles.

Here, we examine the phenotypes of VICs cultured on hydrogel surfaces (2D) and then compare this to VICs encapsulated within the same hydrogel formulation (3D) using traditional metrics alongside microarray experiments to measure global gene expression. As a control, VIC expression in hydrogel matrices is directly contrasted to freshly isolated VICs, as well as VICs cultured on TCPS. This quantitative approach provides insight into many cell functions through the measurement of gene expression levels to demonstrate how *in vitro* culture platforms influence VIC phenotype. Aortic VICs were used for this study as aortic stenosis is the most common valve disease in developed countries [13]. Ultimately, these results should improve the field's understanding of the impact of the design of *in vitro* culture platforms on primary cell phenotype, especially fibroblast and myofibroblast characteristics. Changes in dimensionality can impact a wide range of cell functions, and this knowledge should prove useful in the development of matrices for expanding and culturing cells *ex vivo*, as well as the engineering of cell delivery vehicles for *in vivo* tissue regeneration.

2. Materials and Methods

2.1. VIC isolation and culture

VICs were isolated from aortic valve leaflets of fresh porcine hearts (Hormel) using a previously described protocol [14]. Leaflets were excised and rinsed in Earle's Balanced Salt Solution (Life Technologies) supplemented with 1% penicillin-streptomycin (Life Technologies) and 0.5 ug/mL fungizone (Life Technologies). Leaflets were then incubated in 250 units/mL collagenase type II (Worthington) for 30 min at 37°C and vortexed for 30 s to remove endothelial cells. Next, a second incubation in collagenase was performed for 1 hour at 37°C. Digested leaflets were then vortexed for 2 min and cells were separated from valve debris by filtration through a 100 μm cell strainer. The cell solution was centrifuged and the cell pellet was re-suspended in growth media composed of Media 199 (Life Technologies) supplemented with 15% fetal bovine serum (FBS, Life Technologies), 1% penicillin-streptomycin and 0.5 ug/mL fungizone. Freshly isolated VICs were then used for RNA isolation or plated on tissue culture polystyrene (TCPS, Fisher Scientific) and grown to ~80% confluency before use in experiments. VICs were then seeded on 2D hydrogels at 25,000 cells/cm^2, on TCPS at 12,500 cells/cm^2, or encapsulated in 3D hydrogels at 10 million cells/mL. Hydrogel formulations are described in the next section. Experiments were performed in low-serum (1% FBS) media supplemented with 1% penicillin-streptomycin (Life Technologies) and 0.5 ug/mL fungizone (Life Technologies) in a 37°C incubator with 5% CO_2.

2.2. Synthesis of poly(ethylene glycol)-norbornene (PEG-nb)

8-arm PEG-nb was synthesized as described previously [15,16]. Briefly, stoichiometric amounts of 8-arm PEG (40 kDa, JenKem) and 4-dimethylaminopyridine (Sigma-Aldrich) were dissolved in anhydrous dichloromethane (Sigma-Aldrich). A two-fold excess of each 5-norbornene-2-carboxylic acid (Sigma-Aldrich) and N-N'-diisopropylcarbodiimide (Sigma-Aldrich) were added and reaction vessel was purged with argon. After reacting overnight on ice while stirring, product was precipitated in 4°C ethyl ether (Fisher Scientific). The product was then filtered and dried by vacuum. Next, the PEG-nb was dissolved in water and purified by dialysis. The final product was lyophilized, and the overall end group functionality was characterized by proton nuclear magnetic resonance imaging to confirm >90% functionalization.

2.3. Hydrogel formation and characterization

5 wt% 8-arm PEG-nb was crosslinked with a dithiol-containing, matrix metalloprotease (MMP)-degradable peptide KCGPQGJ.IWGQCK (American Peptide Company, Inc.) and with 2 mM CRGDS adhesive peptide (American Peptide Company, Inc.) at a ratio of 0.55 thiols per norbornene. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was added at a concentration of 1.7 mM. All components were dissolved in phosphate buffered saline (PBS, Life Technologies). Non-stoichiometric ratios of the thiol and –ene functionalities were used to control the final crosslinking density, and ultimately, the gel connectivity and shear modulus to permit cell spreading in cell-laden hydrogels within 48 hours.

2D hydrogels were fabricated on glass coverslips that had been thiolated by vapor deposition of 3-(mercaptopropyl) trimethoxysilane in an 80°C oven to facilitate covalent anchoring of the gels to the coverslips. First, the monomer solution was pipetted onto a SigmaCote (Sigma-Aldrich) treated glass slide and covered with a thiolated coverslip such that the final gel thickness was ~100 μm. For 3D hydrogels, 10 million cells/mL were suspended in the monomer solution, and 29 μL of the cell-monomer solution were added to a mold (5 mm diameter) placed on a SigmaCote treated glass slide. Hydrogels for rheological characterization were formed in the same manner as the 3D hydrogels but without embedded cells. All hydrogels were polymerized by exposure to UV light (~2 mW/cm^2 at 365 nm) for 3 min, as determined by monitoring the evolution of the elastic modulus and its plateau. Gels were then placed in wells containing low-serum media (1% FBS). 2D gels were allowed to swell overnight before seeding with cells. To characterize the materials properties of the hydrogels, the shear elastic moduli (G') of the swollen hydrogels were measured using a DHR3 rheometer (TA Instruments) and a parallel plate geometry. Frequency and strain sweeps were performed to ensure that measurements were taken within the linear regime. Young's modulus was calculated from G' assuming a Poisson's ratio of 0.5 [17].

2.4. Immunostaining for αSMA and f-actin

VICs were fixed overnight in 10% formalin (Sigma-Aldrich) 48 h after seeding or encapsulation. Next, samples were washed with PBS, permeabilized with 0.05% TritonX100 (Fisher Scientific) in PBS, and then treated with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS with Tween20 (Sigma-Aldrich) to prevent non-specific staining. Samples were treated overnight at 4°C with the primary antibody, mouse anti-αSMA (Abcam) diluted 1:200 in the 1% BSA solution. Next, the samples were washed with PBS before incubation with the secondary antibody, goat-anti-mouse AlexaFluor 488 (1:300, Life Technologies) and TRITC-phalloidin (1:300, Sigma-Aldrich) overnight. Finally, cell nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI, Life Technologies). Samples were imaged on a 710 LSM NLO confocal microscope (Zeiss) at 20x magnification with at least 3 fields of view per sample. For 3D samples, slices were taken at 10 μm intervals and images show a maximum intensity projection of 11 slices. The percentage of myofibroblasts in each sample was determined by manually counting the number of cells with and without organized αSMA stress fibers. Three biological replicates were performed per condition.

2.5. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated from VICs 48 h after seeding or encapsulating using TriReagent (Sigma-Aldrich) with two 1-bromo-3-chloropropane (Sigma-Aldrich) extractions and precipitation by 2-propanol (Sigma-Aldrich) according to the manufacturer's instructions. RNA pellets were washed twice with 75% ethanol (Sigma-Aldrich) and re-suspended in water. RNA concentration and quality were assessed with a ND-1000 Nanodrop Spectrophotometer.

For qRT-PCR, cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad) and an Eppendorf Mastercycler. 0.05 ng/μL cDNA, 300 nM custom primers (Illumina), and 10 μL SYBR Green Supermix (Bio-Rad) were combined with water using an EpMotion 5370 (Eppendorf) for a final volume of 20 μL in each well. Expression of αSMA (F: 5'-GCAAACAGGAATACGATGAAGCC-3', R: 5'-

AACACATAGGTAACGAGTCAGAGC-3') was normalized to a reference gene, ribosomal protein L30 (RPL30, F: 5'-GCTGGGGTACAAGCAGACTC-3', R: 5'-AGATTTCCTCAAGGCTGGGC-3'). mRNA levels were determined by running samples on an iCycler (Bio-Rad) and comparing the C_T values for each sample to a standard curve. Three technical replicates were performed for each biological replicate.

2.6. Porcine genome microarrays and analysis

RNA was isolated as described above. Only samples with a concentration greater than 100 ng/μL, 260/280 ≥ 1.8, and 260/230 ≥ 1.8 were used in the microarray experiments. Each condition included 3 biological replicates. RNA quality assessment and microarrays were performed by the Genomics and Microarray Core and University of Colorado at Denver. Samples were hybridized to Affymetrix Porcine Gene 1.0 ST arrays. Data were analyzed using Expression Console (Affymetrix), Transcriptome Analysis Console (Affymetrix), Spotfire (TIBCO), the Database for Annotation, Visualization and Integrated Discovery (DAVID, National Institute of Allergy and Infectious Diseases & National Institute of Health) [18,19], the Panther Classification System (Gene Ontology Consortium) [20,21], and PathwayLinker [22]. Differences were considered significant if there was a fold change greater than 2 or less than −2 and a p-value less than 0.05 unless otherwise specified.

2.7. Statistics

At least 3 biological replicates using cells from separate pools of porcine hearts were performed for each experiment. For analysis of VIC activation by immunostaining, over 100 cells were analyzed per sample. Conditions were compared using one-way ANOVAs in Prism (GraphPad) or the Transcriptome Analysis Console. In figures, error bars represent the standard error of the mean.

3. Results

3.1. VIC αSMA expression changes with culture platform

VICs were cultured on surfaces of hydrogels (2D) or encapsulated within hydrogels (3D) with the same composition and a Young's modulus of 390 Pa. Cells cultured in the gel formulations were then compared to cells cultured on traditional plates (TCPS) (Figure 1). The fibronectin-derived adhesive peptide CRGDS was incorporated to facilitate cell attachment to the matrix. Additionally, an enzymatically-degradable peptide was incorporated within the network to allow cells to locally degrade the matrix to permit cell spreading. The hydrogels were formed with an excess of –ene functionalities to reduce the crosslinking density, and therefore reduce the modulus. This formulation was chosen because the low crosslinking density permits cell spreading in 3D matrices within the timeframe of the experiments (48 hr).

VICs were stained for αSMA and f-actin after 48 hr of culture, and VICs cultured on both TCPS and 2D hydrogel surfaces had elongated morphologies and prominent f-actin stress fibers (Figure 2A-B). In contrast, cells encapsulated within 3D hydrogels were smaller and had a more rounded morphology; however, cellular protrusions extending out into the matrix were observed after 48 hours (Figure 2C). VICs are likely less elongated in 3D because they

must first degrade the local matrix before they can elongate. On TCPS, most VICs exhibited organized αSMA stress fibers, a hallmark of the myofibroblast phenotype. In contrast, VICs cultured on 2D hydrogels or within 3D hydrogels expressed very little αSMA. Quantification of these images shows that hydrogel culture resulted in less than 5% activation, consistent with levels found in healthy aortic valves [5], in contrast to over 75% activation on TCPS (Figure 2D).

αSMA expression was also examined at the mRNA level using qRT-PCR (Figure 2E). Freshly isolated VICs contained a similar level of αSMA mRNA as VICs still residing within the valve, indicating that the isolation procedure did not significantly perturb the VIC fibroblast phenotype towards a myofibroblast. In contrast, culturing VICs on TCPS resulted in an approximately 300-fold increase in αSMA expression. By simply culturing VICs on a softer 20 hydrogel, the αSMA mRNA was reduced by an order of magnitude compared to TCPS, and this was further reduced when the VICs were cultured within a 3D hydrogel where the αSMA level was only ~2-fold higher than the freshly isolated cells. While 2D and 3D hydrogel culture resulted in similar levels of VIC activation as measured by immunostaining for αSMA stress fibers, there was a large difference in αSMA mRNA expression. As one might expect differences at the gene and protein level, this result motivated our interest in better characterizing and quantifying the VIC phenotype through multiple measures.

3.2. VIC expression levels are highly dependent on the culture microenvironment

mRNA expression levels in VICs cultured on TCPS, 2D hydrogels, or within 3D hydrogels were compared to freshly isolated VICs using a porcine DNA microarray [23]. VICs cultured on TCPS had differential expression compared to freshly isolated cells for 3304 probesets (out of 25470), where a difference was defined as a fold change greater than 2 or less than −2 and a p-value less than 0.05 to achieve statistical and biological significance (Figure 3A). The mRNA levels of many genes perturbed by TCPS culture returned to levels consistent with freshly isolated cells when VICs were instead cultured on top of or within soft hydrogels (E~390 Pa). However, one should note that each *in vitro* culture platform resulted in differential expression for many genes compared to freshly isolated VICs. For example, 2076 probe sets had different levels of expression in all 3 *in vitro* conditions, which implies that stimuli present *in vivo* that were not incorporated into these *in vitro* cultures, such as signaling from other cell types and the mechanical deformation of the valve, also have an influence on the transcriptional profile. Here, we focus on the differences that arise from different culture substrates and the dimensionality of the microenvironment.

To assess the overall impact of the culture platform on the transcriptional profile, the data was translated into a complementary cumulative distribution to account for both the number of differentially regulated genes, as well as the magnitude of the changes (Figure 3B). In this representation, the number of genes that had a minimum fold change was plotted against the fold change. While TCPS culture did not result in more differentially expressed genes than hydrogel culture conditions, fold changes of greater magnitude were observed. In fact, the distribution is very similar for all of the culture conditions for low fold changes, but there is a divergence at a fold change of ~32. In the region of large fold changes, there are many

more genes that are highly perturbed in VICs cultured on TCPS compared to either hydrogel condition. Additionally, 3D hydrogel cultures resulted in a somewhat lower incidence of the highly-differential genes than 2D hydrogels.

The tails of this distribution were further examined by extracting out those genes with a fold change greater than 32 or less than −32 and representing the expression levels of these genes in a heat map (Figure 3C). Here, red represents high expression, blue represents low expression, and white represents average expression. This group includes genes related to cytoskeletal organization [α-cardiac muscle actin 1 (ACTC1)], cell-matrix interactions [integrin α8 (ITGA8)], matrix remodeling [MMP9, cartilage oligomeric matrix protein (COMP), keratocan-like protein (LOC100157843)], and chemokine activity (CXCL2, CXCL12). The subset of genes that are highlighted with black lines to the right of the figure were greatly perturbed by TCPS culture, but their expression levels were largely recovered in one or both of the hydrogel platforms.

3.3. Functions and pathways influenced by the culture platform

There are a number of functions and pathways that have been closely tied to VIC-matrix interactions that are important in regulation of VIC phenotype [8,24–29]. Here, we focused on some key functional categories to better understand the major differences between VICs cultured on TCPS, 2D or 3D hydrogels, and fresh isolates. Specifically, heat maps were generated to compare differentially expressed genes involved in cell-matrix interactions, cytoskeletal organization and contractility, TGF-β signaling, and matrix remodeling (Figure 4). The fold change compared to freshly isolated VICs is represented by the color, where warmer colors (red) indicate higher expression, and cooler colors (blue) indicate lower expression. Lighter colors represent expression levels similar to freshly isolated cells, and white indicates an expression level identical to the freshly isolated cells. In this analysis, many genes related to the cell cytoskeleton and contractility are upregulated by plating VICs on TCPS; in contrast, expression levels of many of these upregulated genes are reduced to levels more similar to freshly isolated cells by culture on 2D hydrogels. Interestingly, a number of genes, including ACTA2 (αSMA), ACTC1, tropomyosin 1 (TPM1), and TPM2 remain at elevated levels regardless of the culture environment.

When comparing 2D versus 3D hydrogel cultures, the 3D environment results in expression patterns that are much more similar to freshly isolated VICs. This result suggests that dimensionality may play an important role in VIC cytoskeletal organization. The unnatural polarity and spread morphology observed in 2D cultures may drive changes in protein organization into cytoskeletal structures, as well as changes in the expression of these proteins at the mRNA level. While most of these genes were elevated in 2D, a few [ACTC1, calponin 1 (CNN1), and myosin (MYO, LOC100049650)] had lower expression in 3D hydrogels than in freshly isolated VICs. Perhaps over time, as encapsulated VICs remodel their microenvironment and are able to spread and develop more mature matrix interactions, these levels would become closer to those seen in the fresh isolate.

Focal adhesions are key transducers of the outside-in signaling that enables cells to sense cues from the extracellular matrix and generate forces. In our analysis, focal adhesion genes followed a similar pattern, with TCPS cultures exhibiting large differences in genes related

to focal adhesions while the hydrogel platforms led to expression levels that were similar to freshly isolated VICs. For all of these genes, except ITGB1 and talin 1 (TLN1), 3D hydrogel culture more closely recapitulated the expression levels of freshly isolated VICs than the 2D hydrogel surfaces. Filamin B (FLNB), ITGA8, and vinculin (VCL) were the most upregulated by culture on TCPS. ITGA8 is an integrin that can bind ECM proteins, and FLNB and VCL are both involved in anchoring focal adhesions to the actin cytoskeleton. ITGA2, another integrin, was dramatically downregulated on TCPS. Intuitively, overall upregulation of focal adhesion genes on TCPS might contribute to the ability of VICs to generate additional force and form αSMA stress fibers on this stiff substrate, leading to pathological activation.

Beyond cell-matrix interactions, TGF-β signaling is one of the key signaling pathways that has been implicated in VIC activation and in progression of valve disease. While it may not be intuitive that cytokine signaling would be influenced by the extracellular environment, expression levels of genes in this pathway were dependent on the culture platform. Genes related to TGF-β signaling were both up- and downregulated by culture on TCPS, while both hydrogel culture systems resulted in mostly reduced expression of these genes. The magnitudes of the differences in TGF-β signaling genes were smaller than those reported for the other categories. More of these genes were upregulated on TCPS than in 3D hydrogels, consistent with the higher activation levels and αSMA expression on TCPS; however, TGFB1 does not fit this trend. Interestingly, ITGA8 encodes a subunit of an integrin pair that binds the latent TGF-β1 protein, a step required for the protein to become active [30]. This indicates a mechanism by which a somewhat lower TGFB1 expression level could still lead to greater activation of the TGF-β signaling cascade. Bone morphogenetic proteins are also involved in the TGF-β signaling pathway and are important in the formation of calcific nodules frequently seen in patients with valve disease [31,32]. BMP3 was highly upregulated only by culture on TCPS, indicating a possible shift to a more osteoblast-like phenotype.

Matrix remodeling is a complex series of events that involve cell secretory properties (e.g., deposition of collagen), as well as secretion of proteases and inhibitors of those proteases (e.g., MMPs and TIMPs). When examining several genes related to matrix remodeling, a number of genes, including various collagens, fibrillin 1 (FBN1), heparanase (HPSE), and ADAM metallopeptidase 19 (ADAM19) were elevated in VICs on TCPS, and the expression levels were more consistent with freshly isolated VICs in hydrogel matrices. Interestingly, on TCPS, some of the proteases (HPSE and ADAM19) were even more upregulated than the ECM proteins.

Matrix metalloproteases (MMPs) did not follow a consistent trend, with relative constant expression of MMP2, upregulation of MMP14 only in hydrogel culture, and upregulation of MMP9 in all *in vitro* conditions. Genes encoding laminin α subunits, LAMA2 and LAMA4, had opposite trends, with lower LAMA2 expression and higher LAMA4 expression *in vitro*. This is interesting because it shows that VICs may alter the type of laminin expressed based on their microenvironment, with *in vitro* culture resulting in more of the laminin with a truncated α subunit. Among the *in vitro* conditions, VICs produce more LAMA4 in the 2D

conditions (TCPS and 2D hydrogel) than in 3D hydrogels, perhaps indicating a response to the unnatural 2D environment.

3.4. The influence of dimensionality on VIC phenotype

In addition to better defining the VIC phenotype and studying the effect of culture microenvironment on alteration of this phenotype, we also sought to differentiate the effects of a 2D versus 3D culture environment in maintaining VIC phenotype. In particular, since 3D culture environments render many biological assays more difficult, we sought to quantify critical differences that might arise in VICs that are cultured in an identical matrix, but with a difference in dimensionality.

Using the Affymetrix Transcriptome Analysis Console, we directly compared mRNA levels in VICs seeded on soft hydrogel surfaces (2D) versus those encapsulated within the same hydrogel formulation (3D); 159 differentially expressed probe sets were identified using a cutoff of p-value less than 0.05 and fold change greater than 2 or less than −2. The functional importance of these genes was assessed using DAVID and the Panther gene ontology terms. At a macroscopic level, genes related to cell structure and motility, developmental processes, and proliferation and differentiation were enriched in the population of genes with higher expression in 2D, while transport-related genes were overrepresented in the genes with higher expression in 3D (Figure 5). Changes in cell structure and motility-related gene expression in response to the dimensionality show that observed differences in morphology and migration are not simply a result of physically confining the cells, but are also influenced by the underlying transcriptional profile. Developmental processes were also enriched on 2D hydrogels, indicating that the response to dimensionality may result in activation of similar cell functions as the changing extracellular environment in development. Cell proliferation and differentiation genes were also influenced by hydrogel dimensionality; however, this group includes both genes that promote [ephrin type A receptor 4 (EPHA4), KIT] and inhibit [inhibin beta A (INHBA)] proliferation. Collectively, this assessment shows that while there are differences in the regulation of proliferation, it is difficult to elucidate the overall influence of dimensionality on proliferation based on these results alone. Genes with higher expression in encapsulated VICs were associated with transport of various molecules, including ions and lipids, in and out of the cell. The differences in these membrane channels are likely a result of the unnatural polarity induced in the VICs when they are seeded on a 2D surface.

As not all phenotypic changes can be observed at the mRNA level, PathwayLinker was used to map differentially expressed genes (2D vs. 3D hydrogels), as well as the proteins that directly interact with the translated transcripts (Supplementary Figure 1). While pathways of interest can be analyzed individually, this approach can obscure many complex ways in which signaling pathways interact. For example, tissue plasminogen activator (PLAT) is a protein that regulates tissue remodeling [33], but Supplementary Figure 1 shows that PLAT interacts with proteins involved in cell adhesion signaling, actin cytoskeleton organization, and TGF-β signaling. These interactions demonstrate the difficulty of independently studying any one of these pathways in isolation. Another gene of particular interest is KIT, a kinase involved in activation of several signaling pathways, including AKT, RAS, and

MAPK [34]. The high level of connectivity to other genes of interest suggests that this kinase could be a key regulator of the VIC response to dimensionality.

4. Discussion

VICs are important regulators of valve ECM, but pathological VIC activation leads to excessive collagen deposition, a disorganized matrix, and valve fibrosis [6]. Mechanisms contributing to this disease state are still being elucidated, and there are no clinical treatments to slow or reverse valve fibrosis [4]. While there are no large animal models of valve disease that extend beyond the stage of early valve sclerosis, several research groups have developed mouse models of valve disease that often involve a combination of genetic and diet modifications [35,36]. While much is learned from these animal models, *in vitro* models of valve cells would provide complementary information and allow for more detailed hypothesis testing to support or help refine ideas related to targets for reversing fibrosis. However, traditional culture of VICs primarily leads to a myofibroblast phenotype [8], and it is difficult, at best, to characterize the quiescent VIC fibroblasts or study conditions that would lead to reversal of the pathogenic VIC myofibroblast population. Part of the complexity is that VICs receive cues from the extracellular matrix that cannot be recapitulated by culture on TCPS, which has a stiffness that is greater than six orders of magnitude higher than valve tissue.

In this study, soft (E = 390 Pa) hydrogels were used as a culture platform to study VICs under conditions in which they are not always activated to the myofibroblast phenotype, as seen when using traditional cell culture methods [8]. Specifically, we used PEG-based hydrogels with an MMP-degradable crosslinking peptide and the fibronectin-derived CRGDS peptide to mimic aspects of the extracellular matrix by allowing local cell remodeling, spreading, and adhesion. This peptide-modified synthetic polymer system enables the controlled presentation of both mechanical and biochemical matrix cues. The gels were formed via a cytocompatible photoinitiated thiol-ene polymerization [16]. The modulus of this formulation, 390 Pa, is softer than the moduli typically reported for aortic valves. Reported values range from around 1400 kPa up to tens of MPa due to the anisotropic and heterogeneous nature of the valve tissue, which leads to a highly non-linear stress-strain relationship [37–40]. However, the selected hydrogel composition and crosslinking density allowed for the use of the exact same material in both the 2D and 3D cell experiments, since the low crosslinking density permits cell spreading in 3D within 48 hours. While the local modulus around the VICs may vary over time due to cellular remodeling of the matrix, these PEG-based hydrogels maintain consistent bulk mechanical properties throughout the 48 hour culture time, while VICs encapsulated within collagen or Matrigel can contract the matrix and dramatically alter the material properties [41]. In this study, only one adhesive ligand (CRGDS) was tested, but others have shown that adhesive peptides derived from different ECM proteins can influence VIC secretory properties and ECM deposition [26]. Investigating the influence of various adhesive ligand sequences on VIC gene expression would likely lead to identification of different integrin signaling cascades and aid in the design of matrices for valve regeneration. Here, we chose to focus on the influence of dimensionality of VIC function, as previous work has demonstrated that

dimensionality has a greater impact on embryonic stem cell gene expression than specific ECM proteins (e.g., collagen vs. gelatin) [42].

Many efforts to elucidate the effects of dimensionality compare cells in native tissue or aggregates to those cultured on TCPS [43–46]. These experiments are certainly pioneering and relevant, but many variables change from native ECM to TCPS, in addition to the dimensionality (e.g., modulus, chemical composition). Because of this, one must use caution when drawing conclusions about the importance of dimensionality, and this motivated our experimental design to use a highly controlled hydrogel matrix in this study. Consistent with previous studies [8], we have shown that VICs cultured on TCPS have a dramatically altered phenotype compared to freshly isolated cells. In addition to an increased proportion of cells expressing the myofibroblast phenotype (Figure 2), culturing VICs on TCPS results in significant changes in expression levels for 3304 probe sets. In addition, genes related to a number of critical biological processes, including cell structure and transport (Figure 5), were dependent of the dimensionality in which the VICs were cultured, even though VIC populations in both 2D and 3D hydrogel matrices consistently largely of quiescent fibroblasts.

In this study, we found that each of the *in vitro* culture conditions resulted in some differences in gene expression levels compared to the freshly isolated cells (Figure 3A). It is not surprising that a number of genes would always be different in an *in vitro* culture system, as there are a number of complex stimuli that influence VICs *in vivo*. For example, none of the culture platforms include any cell types other than the VICs. Specifically, valvular endothelial cells have been shown to play a role in regulating VIC phenotype [47–50]. Cells involved in the inflammatory response, such as macrophages and T lymphocytes, have also been excluded from this study. This is not necessarily a disadvantage though, because excluding other cell types allows one to focus on the effects of matrix mechanics and eliminate confounding interactions that could occur in a model with added complexity. Another simplification in these *in vitro* studies is the mechanically static nature of all of the culture platforms. *In vivo*, the valve is exposed to bending, stretching, and blood flow. These stresses on the cells can also influence their phenotype and these dynamics warrant future study, but we expect that using static culture platforms introduces some of the differences in gene expression that are common to each culture method.

While both 2D and 3D hydrogel conditions had a greater number of differentially expressed genes than TCPS culture, using the standard criteria of fold change greater than 2 and p-value less than 0.05, the distribution of the fold changes shows that TCPS leads to a population of genes that have very high fold changes not seen in hydrogel culture (Figure 3B). Additionally, this distribution reveals that culture in 3D hydrogels results in slightly fewer genes with extreme fold changes than 2D hydrogel substrates. Many of the highly perturbed genes, including ACTC1, NR4A2, ITGA8, and FOS, returned to levels more consistent with freshly isolated VICs in one or both of the hydrogel culture platforms.

Culture on TCPS dramatically changes VIC phenotype. Conventional VIC characterization methods may suggest that TCPS represents a fibrotic microenvironment, but our results show that it represents something more extreme than typical disease. When the cells are

characterized simply by the most common methods of αSMA quantification or staining for αSMA stress fibers, these cells appear to be acting similarly to diseased VICs, with higher levels of αSMA and a large fraction of cells exhibiting the myofibroblast phenotype. However, upon a more global examination of gene expression levels, we have seen that culturing VICs on TCPS results in more changes in gene expression than exist when comparing healthy and stenotic aortic valves in a similar experiment. Bosse *et al.* found that 1002 of 54675 probe sets showed significant differences between expression in healthy and stenotic valves [31]. When the same criteria are applied to this study, 6316 out of 25470 probe sets showed differences between freshly isolated VICs and VICs cultured on TCPS. While results obtained from different array chips are not directly comparable, this significant difference supports the idea that even though VICs cultured on TCPS resemble diseased VICs more than healthy VICs, TCPS may still be a poor model for valve disease because so many additional genes are also perturbed. For example, if one were to screen for inhibitors of pathogenic VIC myofibroblasts on TCPS, certain candidates may be missed as the VIC context is important and can influence receptor signaling, concentration profiles, and mechanotransduction.

This study demonstrates that the choice of culture platform (TCPS, 2D hydrogel, or 3D hydrogel) can influence many genes and cellular functions associated with VIC activation and valve disease, such as cytoskeletal organization and contractility, focal adhesions, TGF-β signaling, and matrix remodeling. Overall, these findings demonstrate the importance of choosing a culture platform relevant to the pathway or function of interest to ensure that critical genes are not vastly up- or downregulated. Genes related to cytoskeletal organization were expressed at different levels in each of the culture conditions. 3D hydrogels resulted in an expression profile most similar to freshly isolated VICs, and 2D hydrogels did not perturb expression levels as much as TCPS. This indicates that both the dimensionality and the substrate modulus are influencing cytoskeletal organization. This same finding held true for focal adhesion genes, where 3D hydrogel culture best recapitulated the expression levels in freshly isolated VICs. As focal adhesions are the key structures that mediate the interaction between cells and the ECM, it is interesting to note that VICs in different culture conditions are not only interacting with different environments, but are also changing the way in which they mediate their interactions with their environment. In contrast, TGF-β signaling genes had similar levels of expression in both 2D and 3D hydrogels, indicating that dimensionality has less of an influence on this pathway. The modulus of the matrix has been implicated in the ability of fibroblasts to activate TGF-β1 from its latent form [51], so the matrix modulus may play a larger role in TGF-β signaling.

The up- or downregulation of many matrix remodeling genes was unpredictable, with TCPS increasing the level of some ECM genes (LAMA4, many collagens) and decreasing the expression of others (LAMA2, SHAS2). Similarly, proteases were both upregulated (MMP9, HPSE) and downregulated (MMP2). It is perhaps counterintuitive that the proteases HPSE and ADAM19 were more highly expressed in cells on a 2D surface than they are in cells entrapped within a 3D matrix. The matrix stiffness may play a role, but does not entirely account for this difference. It is possible that this result is a function of the relatively short (48 hr) time point at which these samples were collected, and further

investigation as a function of time, when more extensive matrix remodeling has occurred, should provide added insight. From this data, it is difficult to conclude whether this would result in overall increases in ECM deposition, yet it seems clear that this transcriptional profile of matrix remodeling genes would result in different tissue architecture. For the most part, expression levels in hydrogel cultures were more similar to those seen in freshly isolated VICs; however, LAMA2 and MMP9 remained highly perturbed in all conditions.

While 3D hydrogel culture resulted in expression levels that were slightly more similar to freshly isolated VICs than the 2D hydrogel culture, there were not dramatic differences in expression patterns of these matrix remodeling genes. The physical remodeling of the matrix must be highly dependent on dimensionality as 3D environments necessitate cell degradation of the matrix to spread or migrate, and the dimensionality of the environment would almost certainly influence the ability of the cells to incorporate secreted ECM proteins into the surrounding matrix. These changes may be more apparent at a later time point than the 48 hour culture studied here, as most studies of tissue engineering of a valve look at time points weeks or months after seeding [40,52]. With respect to this, microarray data presenting expression levels of ECM-related genes could be complemented by studies focused on histological staining of gels or thorough quantitative assays to measure ECM proteins.

The global view comparing RNA levels in different culture platforms to freshly isolated cells also provides valuable insight into the choice of reference or “housekeeping” genes. Traditionally, internal standards have been chosen because they did not appear to vary after treatment with various drugs or other molecules. A number of genes have become commonly accepted internal standards, including GAPDH, ACTB (β-actin), and genes encoding for ribosomal proteins. While the expression levels of these genes may not vary significantly among experiments on TCPS, these microarray results show that some of these genes may be a poor choice when comparing *in vitro* to *in vivo* experiments (Supplementary Figure 2). Specifically, both GAPDH and ACTB are expressed at significantly different levels on TCPS compared to freshly isolated VICs. Many ribosomal genes were much more consistently expressed, with RPS18, RPL30, and RPL32 not exhibiting any significant differences between any conditions. These findings are generally consistent with a previous study comparing thousands of human and mouse arrays, which found high variability in ACTB, GAPDH, HPRT1, and B2M and instead recommended mostly genes encoding ribosomal proteins [53].

Collectively, this study provides quantitative measures of the importance of the culture platform on VIC behavior *in vitro*. Building on these findings, future studies to identify pathways that might be leveraged to prevent or reverse valve disease could be enabled by exploiting soft, 3D hydrogel environments that better recapitulate native gene expression levels. Such studies are difficult to perform on TCPS, as it significantly perturbs baseline expression levels of key myofibroblast markers and might lead to false negatives when screening for useful therapeutics. Additionally, signaling cascades initiated by therapeutics may have important interactions with mechanotransduction pathways [54]. While 3D hydrogel culture led to transcriptional profiles more similar to freshly isolated VICs, there are still many differences. In order to study a pathway of interest that involves many of these

differentially regulated genes, it may be worthwhile to develop new culture platforms that better recapitulate the desired pathway by modifying matrix characteristics, such as the modulus or the functionalization with peptides, or by focusing on other elements present *in vivo*, such as valvular endothelial cells or cyclic strain. Many of these questions could be addressed by modifying the materials presented here to incorporate additional complexity, as PEG hydrogels are amenable to co-culture with valvular endothelial cells [50], integration into microfluidic devices [55], and culture with bioreactors [56].

5. Conclusions

Microarray analysis of VIC transcriptional profiles reveals and quantifies how cell phenotype *in vitro* is highly dependent on the culture platform. This global transcriptome analysis shows that TCPS culture results in higher-magnitude changes to gene expression levels than either 2D or 3D culture. Culture on TCPS greatly perturbed many genes related to cytoskeletal organization and contractility, focal adhesions, TGF-β signaling, and matrix remodeling, demonstrating the importance of considering which culture platforms are appropriate for studying specific cell functions or pathways. While characterizing the percentage of VICs expressing the myofibroblast or fibroblast phenotype is a useful metric, we have demonstrated that populations of VICs with the same activation levels can have dramatically different phenotypes, which emphasizes the importance of using multiple measures to characterize cell response to microenvironmental cues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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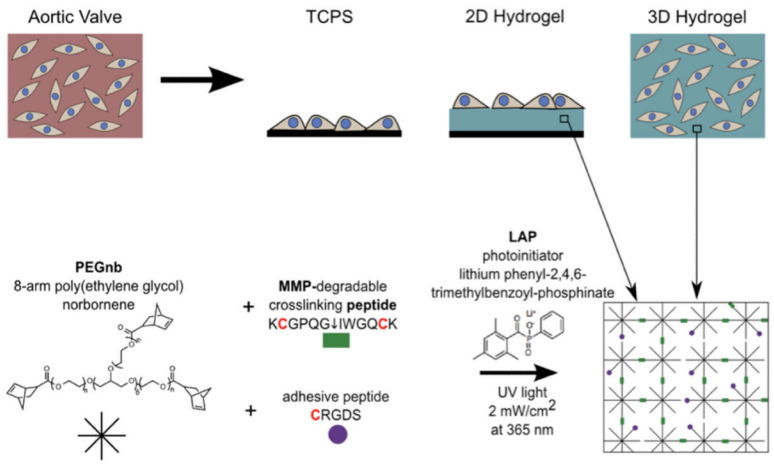

Figure 1. VIC culture platforms

VICs were isolated from porcine aortic valves and either saved for RNA isolation or plated and expanded to ~80% confluency. Then, cells were seeded on TCPS or 2D hydrogels or encapsulated within 3D hydrogels of the same formulation. Hydrogel matrices were formed by a photointiated thio-ene polymerization of 8-arm PEGnb (40 kDa) and an MMP-degradable peptide. The cleavage site is indicated by an arrow. A fibronectin-derived peptide, CRGDS, was incorporated to facilitate cell adhesion to the hydrogels. The cysteines indicated in red react with the norbornene groups on the PEG through a. thiol-ene, photoclick reaction.

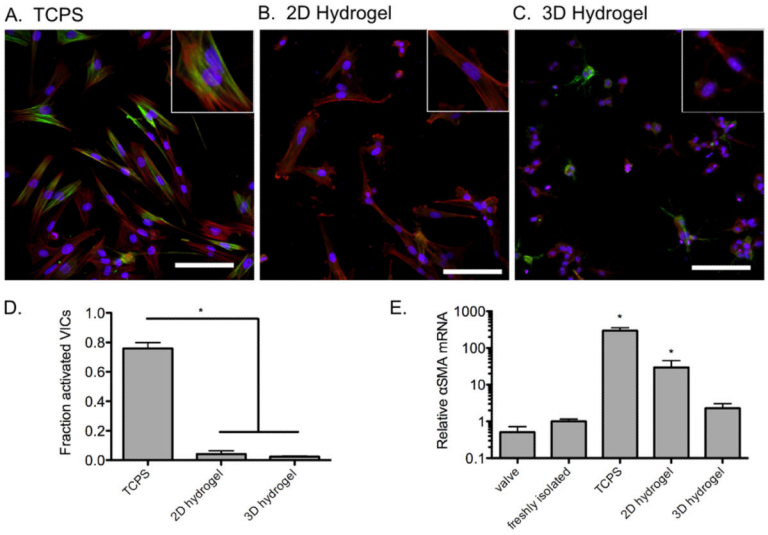

Figure 2. Characterization of VIC activation and αSMA expression

VICs A) on TCPS, B) on 2D hydrogels, and C) within 3D hydrogels were immunstained for αSMA (green), f-actin (red), and nuclei (blue). Scale bar = 100 μm. D) Images were quantified by counting the fraction of cells exhibiting αSMA stress fibers, a hallmark of the activated myofibroblast phenotype. On TCPS, most VICs were activated. With either the 2D or 3D hydrogel culture platforms, very low levels of activation were observed. * indicates $p<0.05$. E) qRT-PCR demonstrated that αSMA mRNA was greatly increased when VICs were cultured on TCPS compared to cells within the valve or to freshly isolated cells. When VICs were instead cultured on 2D hydrogels, there was an order of magnitude reduction in αSMA mRNA. In 3D hydrogels, the αSMA mRNA level was further reduced and was not significantly higher the freshly isolated VICs. * indicates $p<0.05$ compared to freshly isolated VICs.

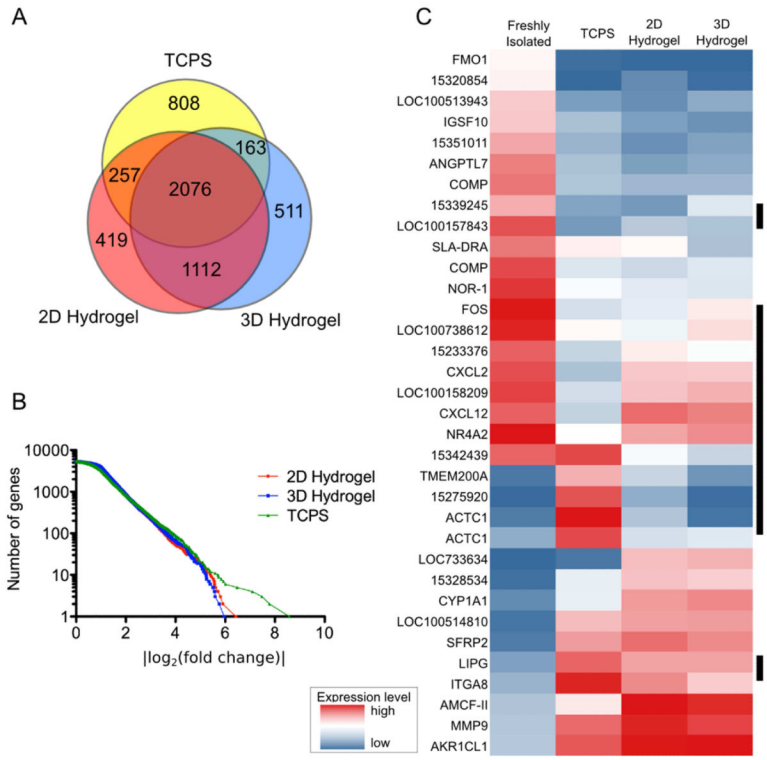

Figure 3. Culture platform directs VIC transcriptional profile

A) Venn diagram showing the number of genes in each culture condition with expression levels different than those seen in freshly isolated VICs. The center region of 2076 genes were differentially regulated in all *in vitro* conditions. B) Complementary cumulative distribution showing the number of genes with up to the given difference in RMA-normalized bi-weight averages. The tail on the TCPS distribution indicates that for a number of genes, expression levels are extremely affected by culture on TCPS. C) Heat map showing the expression levels of all genes with a $\log_2$(fold change) greater than 5 or less than −5 in any culture condition compared to freshly isolated cells and ordered by hierarchical clustering. Red represents high expression, blue represents low expression, and white represents an average level of expression. Genes with expression levels that were drastically altered by TCPS culture but recovered with culture on/in 2D or 3D hydrogels are marked on the side by the black lines.

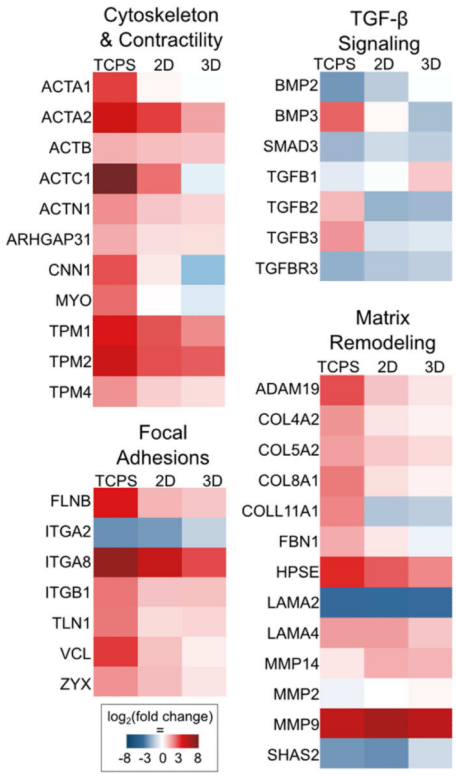

Figure 4. Culture platform influences functions critical to valve disease

Heat maps showing gene expression of differentially expressed genes for selected cell functions: cytoskeletal organization and contractility, TGF-β signaling, focal adhesions, and matrix remodeling. Fold change is represented by color on a log scale. Boxes closer to white represent expression levels similar to the freshly isolated VICs. White = same expression levels as freshly isolated VICs, blue = downregulated, red = upregulated.

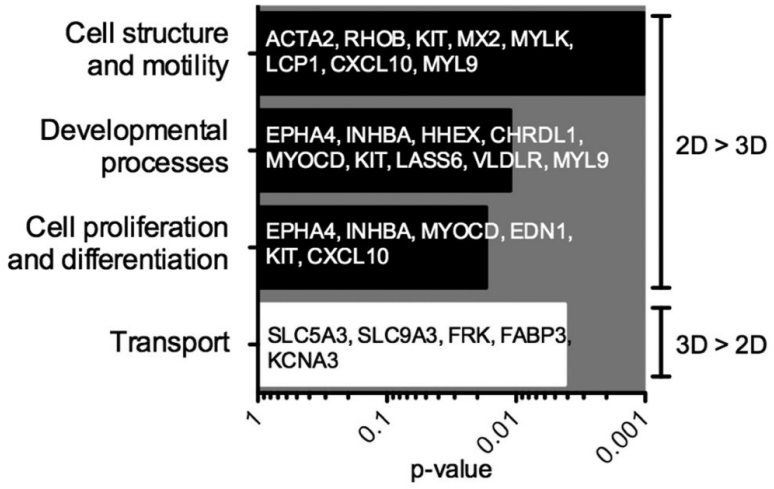

Figure 5. Biological processes influenced by dimensionality

Analysis with DAVID of biological processes (Panther database) that are enriched with genes that are differentially expressed in 2D vs. 3D hydrogels. Genes involved in cell structure and motility, developmental processes, and cell proliferation and differentiation had increased expression on 2D hydrogels, while genes associated with transport had higher expression in 3D hydrogels.