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Regulation of Valvular Interstitial Cell Mineralization by Components of the Extracellular Matrix

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Abstract

The composition of the extracellular matrix (ECM) is believed to play a role in heart valve disease, and is highly relevant to the design of heart valve tissue engineering scaffolds, yet the interaction of valvular interstitial cells (VICs) with the ECM environment has not been well characterized. Thus, the transformation of VICs to an osteoblast-like phenotype was quantified in VICs cultured on different types of ECM coatings. The results show that the number and size of calcific nodules formed in VIC cultures, as well as the expression of the mineralization markers alkaline phosphatase (ALP) and CBFA1, were highly dependent upon the composition of the culture surface. In fact, VICs cultured on certain ECM components, namely collagen and fibronectin, were resistant to calcification, even upon treatment with several mineralization-inducing growth factors. Meanwhile, cultures of VICs on fibrin, laminin, and heparin coatings not only had a high number of calcified nodules, but also elevated levels of ALP and CBFA1. Nodule composition analysis revealed the presence of multiple types of mineralization, including hydroxyapatite. Although apoptotic and necrotic cells were more concentrated in nodules than in other parts of the VIC cultures, the nodules contained a strong majority population of viable cells. By demonstrating this ECM-dependence of VIC calcification, we aim to identify appropriate biomaterial environments for heart valve tissue engineering as well as elucidate mechanisms of valvular disease.

Keywords

valvular interstitial cells; calcification; biomaterials; extracellular matrix

Introduction

The aortic valve is the most extensively studied, most frequently diseased, and most widely transplanted valve in the heart^{1, 2}. While heart valves are relatively small, thin structures (on the order of a few hundred microns thick), their composition is surprisingly complex. Heart valves consist of layers of histologically distinct tissue, with each layer containing a specific distribution of extracellular matrix (ECM) components which include glycosaminoglycans (GAGs), elastin, and collagen³. There are two general cell populations within valves; these

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are valvular endothelial cells, which provide a non-thrombogenic external lining, and valvular interstitial cells (VICs), which comprise the bulk of the valve cell population⁴. The adaptive, complex, and dynamic structure of heart valves can be primarily attributed to the VICs, which are responsible for valve ECM production as they constantly remodel and repair the valve⁴. The organization and relative proportions of the valve matrix are paramount to valve function, thus emphasizing the importance of these interstitial cells.

Primary valvular dysfunction may occur through a variety of mechanisms. Its causes include congenital defects, damage due to diseases such as rheumatic fever and syphilis or infections such as endocarditis, and onset of calcific stenosis as a result of aging⁵. Calcification is the major source of failure of both native valves and tissue valve replacements⁶. Specifically, non-rheumatic calcific aortic stenosis leads to the fibrotic thickening and calcification of the heart valve, and is the most common heart valve disorder in developed countries. Calcified heart valves are rich in activated VICs (known as myofibroblasts) in addition to numerous osteogenic growth factors and cytokines, including bone morphogenetic proteins (BMPs), transforming growth factor-beta1 (TGF- β 1), and tumor necrosis factor-alpha (TNF- α)⁶⁻⁹. TGF- β 1 in particular has been shown to play a key role in valve calcification^{6, 7}. TGF- β 1 induces an activated, myofibroblast-like phenotype in VICs, as evidenced by an increase in alpha-smooth muscle actin (α -SMA) expression and stress fiber formation, often leading to pathological remodeling of the ECM by VICs *in vitro*¹⁰.

Disruption of the ECM environment is frequently found in diseased heart valves, yet the role of ECM components in VIC function and dysfunction remains poorly understood. It has been documented that osteogenic differentiation genes markers (i.e. alkaline phosphatase, ??) are expressed in explanted calcified aortic valves, calcified bioprosthetic valves, and myxomatous ("floppy") mitral valves¹¹⁻¹³. However, many of these data come from end-point analyses of diseased valves. The literature contains only a handful of studies that involve the characterization of *in vitro* calcific nodule formation by VICs. Moreover, there is significant disagreement amongst these studies on the topic of whether VICs spontaneously calcify in culture^{6, 14, 15}, possibly due to different culture substrates being used in each study. In this publication, we describe a controlled study of how specific elements of the VIC environment impact (i.e. promote or inhibit) the emergence of this osteoblast-like phenotype in order to address these issues and provide useful information for heart valve tissue engineering.

Because valvular disease is often accompanied by grossly altered ECM composition and arrangement¹⁶, we have hypothesized that components of the ECM may actively regulate VIC function and dysfunction. The ECM molecules explored in this study represent both components found in native heart valves (i.e. collagen, fibronectin, laminin), as well as those currently being explored by other groups for use in heart valve tissue engineering (i.e. fibrin [ref] and heparin [ref]). We propose that characterization of the role of individual ECM components in regulating VIC culture calcification will help us to: (1) develop appropriate biomaterials for valve regeneration, (2) better understand mechanisms of valve disease and calcification, and (3) identify targets for treatment or prevention of valve dysfunction. Thus, in the following sections, we describe the characterization of VIC culture calcification on 2-dimensional surfaces modified with an array of extracellular matrix components.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

ECM coatings

Tissue culture polystyrene (TCPS) wells of 24-well plates were coated with collagen type I (Coll, BD Biosciences, San Jose, CA; 0.08, 0.8, 8 µg/ml), fibronectin (FN, 0.2, 2, 20 µg/ml), laminin (LN, BD Biosciences; 0.8, 8, 12 µg/ml), heparin (Hep, 10, 200 µg/ml), or left uncoated. All coatings were prepared in 50 mM bicarbonate coating buffer (pH 8.5; except for LN, prepared in phosphate buffered saline, PBS) and incubated at 37°C overnight. Fibrin (Fb) coatings were prepared by adsorbing fibrinogen to TCPS (0.01, 0.1, 1 mg/ml) overnight at 4°C temp, reacting with thrombin (0.258, 2.58, and 25.8 U/ml for respective Fb coatings), and rinsing three times with PBS-T (PBS with 0.1% Tween-20) over the course of one hour. The coating density was quantified on separate plates using the NanoOrange protein assay (Invitrogen Corp., Carlsbad, CA), or GAG assay as previously described¹⁷. All 2-D ECM concentrations are reported herein as their final (quantified) coating density.

VIC Culture and Seeding

VICs were isolated from porcine aortic valves (Hormel, Inc., Austin, MN) by collagenase digestion as previously described¹⁸ and cultured in growth medium (15% FBS and 2% penicillin/streptomycin in Medium 199) at 37°C, 5% CO₂ for 2–4 passages. Following trypsinization, VICs were seeded on ECM-coated and control surfaces described above at a density of 50,000 cells/cm² and cultured in low-serum (1% FBS) medium for the duration of the calcification experiments. These experiments were also repeated in the presence of recombinant human TGF-β1 (0.5 ng/ml), bone morphogenetic protein-2 (BMP-2; 0.1 µg/ml), and BMP-7 (0.02 µg/ml; all from Peprotech, Inc., Rocky Hill, NJ), which were added to cultures every 48 hours for five days.

Characterization of VIC Culture Calcification

After five days of culture, cells were fixed in 10% neutral buffered formalin and stained with 2% Alizarin Red S to clearly visualize and quantify calcific nodule formation. Photomicrographs of cultures were obtained using an Olympus IX-51 microscope with Hamamatsu 285 digital camera and Compix imaging software (Compix Inc., Sewickley, PA). Nodule size analysis was performed using NIH ImageJ Software. Every calcific nodule in every well in each of three duplicate experiments was measured in area. Standard immunocytochemical methods were then used to qualitatively detect alpha-smooth muscle actin (α-SMA) expression. Briefly, cells were permeabilized with 0.1% Triton X-100, incubated with an antibody to α-SMA (monoclonal, mouse, clone 1A4), goat anti-mouse AlexaFluor 488 (Invitrogen), counterstained with DAPI, and photographed.

SEM-EDS (scanning electron microscopy with energy-dispersive x-ray spectroscopy, JEOL JSM-6100) was used to characterize the composition of the calcific nodules. VICs were grown on plain or ECM-coated polystyrene coverslips for 5 days, fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer overnight, followed by dehydration via sequential ethanol rinses, immersion and subsequent drying in HMDS (1,1,1,3,3,3-

hexamethyldisilazane;¹⁹), and then carbon coating. The molar ratio of calcium to phosphorus (Ca/P) was calculated using SEM-EDS data, and this molar ratio is indicative of what type of mineralization is present in the sample²⁰ (i.e. hydroxyapatite = $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 = 10\text{Ca}/6\text{P} = 1.667$). EDS analysis was performed on six nodules in each well, generating elemental composition information for each nodule. The percent of all of the well's nodules identified as a certain type of mineralization was calculated to generate information about the characteristics of the calcified cultures.

Characterization of Mineralization Markers

With respect to studying the osteoblast-like phenotype, we have focused on two markers of osteogenic activity – alkaline phosphatase (ALP) and core binding factor alpha-1 (CBFa1). ALP is commonly used as an indicator of osteoblastic activity, while CBFa1 is a transcription factor involved in osteoblast differentiation. Prior to performing analysis of mineralization markers, DNA was quantified using the CyQuant assay (Invitrogen) per kit instructions, in combination with λ -DNA to generate a standard curve. The amount of alkaline phosphatase (ALP) present in the VIC cultures was assessed via the addition of an ALP chromogenic substrate solution (p-nitrophenyl phosphate – 1-step-pNPP solution, Pierce, Rockford, IL) to wells containing VICs cultured for five days on different ECM coatings and control surfaces. The intensity of the colorimetric response was read at 405 nm (Synergy HT Plate Reader, BioTek Instruments, Winooski, VT) and normalized to sample DNA content. Expression of CBFa1 was analyzed via an in situ ELISA (monoclonal anti-CBFa1 from Alpha Diagnostics, San Antonio TX), which was a modification of a recently published CBFa1 immunostaining protocol²¹. Following the addition of goat anti-rabbit HRP, 1-step Turbo TMB (3,3',5,5'-tetramethylbenzidine; Pierce) chromogen solution, and H_2SO_4 stop solution, sample absorbances (correlating to CBFa1 presence) were read at 405 nm using a plate reader, and normalized to DNA content.

Statistical Analysis

Data were compared using two-tailed, unpaired t-tests or one-way analysis of variance (ANOVA) with Tukey's HSD post-test where indicated. P values less than or equal to 0.05 were considered statistically significant. Data are presented as mean $\pm$ standard deviation.

Results

Type of ECM coating significantly impacts quantity of calcified nodules

The results in Figures 1, 2 and 3 clearly demonstrate that VIC calcification, morphology, and α -SMA expression were highly dependent upon the type of ECM component on which they were cultured. Specifically, as demonstrated in Figure 1, extensive calcification was observed in VIC cultures on TCPS and high concentrations of fibrin, heparin, and laminin. The number of nodules on the highest fibrin concentration was statistically higher than on any other surface ($p < 0.05$). It should also be emphasized that formation of calcific nodules occurred spontaneously in these cultures, without the addition of special mineralization medium or other exogenous factors. Meanwhile, little to no calcification was observed on several other ECMs, namely collagen and fibronectin. As collagen concentration decreased (i.e. the surface became more like TCPS), there was an increase in calcification ($p < 0.01$).

Thus, the presence of collagen or fibronectin appeared to inhibit the formation of calcified nodules in the VIC cultures, and as the concentration of these components decreased, more nodules formed. Conversely, in the cases of laminin, heparin, and fibrin, the calcification of cultures on these surfaces tended to increase with increasing ECM coating density, thus implying that the presence of these factors were actively promoting, or at least becoming more permissive of the calcification. For fibrin in particular, the trend of nodule number increasing with surface concentration was significant ($p < 0.02$).

To further explore these unique findings, particularly the apparent suppression of calcification on collagen and fibronectin, calcification experiments were repeated in VIC cultures treated with three different growth factors (TGF- β 1, BMP-2, and BMP-7) that are well-known potent stimulators of mineralization and osteogenesis^{7, 10, 22}. The purpose of this experiment was to evaluate the robustness of the calcification inhibition found upon collagen and FN surfaces – i.e. whether the results shown in Figure 1 would still hold when the VICs on these surfaces were faced with an environment where mineralization is actively promoted, since the previous experiments had been performed in the absence of any forced mineralization induction or mineralization medium. As observed in Figure 2, treatment of VICs cultured on either collagen or FN with factors that induce mineralization still did not lead to culture calcification, which is a very noteworthy finding that implies active inhibition of calcification by these ECM components. Meanwhile, calcific nodule counts on the control surface demonstrated that the VICs cultured on a surface that permitted calcification in Figure 1 do indeed respond to these growth factors with significant (as much as 3-fold) increases in calcification.

Representative photomicrographs of VIC cultures on four of the surfaces examined are presented in Figure 3. In contrast to the elongated VICs in homogeneous culture seen on the collagen-coated materials, VICs on ‘pro-calcific’ surfaces migrate to form ridges, upon which calcific nodules are formed. In the left panels of photomicrographs, it can be seen that very few VICs on collagen stained positively for α -SMA, while the majority of VICs on laminin, TCPS, and fibrin were α -SMA-positive. Thus, a positive correlation between α -SMA expression and culture calcification is implied.

Calcified Nodule Characteristics Vary with ECM Coating

Amongst conditions where nodule formation was observed, the average nodule size also significantly differed with the type of ECM coating, as illustrated in Figure 4a. The average nodule size on TCPS was significantly larger than nodules on all but one other material (collagen $0.2 \mu\text{g}/\text{cm}^2$). With the exception of TCPS having high values for both nodule count (Figure 1) and average nodule area (Figure 4a), few similarities exist between nodule number and average nodule size data for the different surfaces. The size of nodules on fibrin-coated surfaces decreased with increasing fibrin concentration ($p < 0.05$), which is the opposite trend as that observed for nodule number on fibrin. Thus, with increasing fibrin concentration, cultures progress from a moderate number of very large nodules to a high number of small nodules. Due to this apparent balance between nodule number and nodule size, the sum of the calcified areas per well was quantified for each coating condition, and these data are shown in Figure 4b. The results for total calcified area exhibit some

similarities to the trends observed in Figure 1, namely the very low calcification of collagen and fibronectin, and increased calcification of fibrin with increasing fibrin density.

Sample analysis using SEM-EDS yielded information regarding the composition of nodules found in VIC cultures. Mineralization goes through multiple stages of maturation, listed in order of increasing maturity in Figure 5. As observed in Figure 5, nodules at all stages of mineralization maturity were found in VIC cultures on TCPS, with significantly more dicalcium phosphate dihydrate (the least mature type of mineralization) than octacalcium phosphate, tricalcium phosphate, or hydroxyapatite ($p < 0.02$). It is also noteworthy that VIC cultures progressed to form measurable amounts of hydroxyapatite in a span of only 5 days of *in vitro* culture in the absence of special mineralization medium.

ECM coating affects the expression of osteoblast-like mineralization markers

As seen in Figures 6a and 6b, the production of ALP and CBFA1 in VIC cultures greatly differed with the ECM coating upon which the VICs are cultured. Notably, increasing concentrations of fibrin and heparin were associated with large increases in ALP and CBFA1 ($p < 0.01$ by ANOVA for fibrin CBFA1). Meanwhile, ALP and CBFA1 production by VICs cultured on collagen and fibronectin coatings was very low. As evidenced by the overlay of ALP, CBFA1, and calcific nodule quantification graphs in Figure 6c, there was a strong correlation between ALP activity, CBFA1 expression, and the number of calcific nodules per well. These data indicate that the appearance of nodules in our VIC cultures is associated with expression of multiple osteoblast markers, and that this VIC phenotypic change is regulated by the type of ECM upon which the cells are cultured.

Discussion

The mineralization of vascular smooth muscle cells (SMCs) has been extensively characterized in the literature in order to better understand the pathogenesis of atherosclerosis and vascular disease²³. However, there is relatively little information in the literature regarding the calcification of valvular cell cultures. Because the structure of heart valves and functions of valvular cells differ considerably from those of vessels and vascular smooth muscle cells, studies that explicitly study valvular components (i.e. VICs) are necessary in order to better understand valvular stenosis and to design biomaterials that are specifically suited for valvular tissue engineering. Several recent publications have described factors that contribute to VIC activation to a myofibroblast phenotype^{10, 24}. VIC activation and calcification are widely hypothesized to be linked, although the nature of their relationship is not entirely clear. Myofibroblasts are crucial elements in normal valves, and VIC activation to a myofibroblast phenotype is a natural part of valvular repair [ref]. It is believed that prolonged persistence of a myofibroblast phenotype contributes to pathological outcomes such as calcification. Thus, since mere α -SMA expression appears to be a rather complicated predictor of VIC (dys)function, we have focused our analysis upon the study of an outcome that is obviously pathological, namely the formation of calcified nodules and expression of an osteogenic markers. Emergence of an osteoblast-like phenotype in VICs has been recently documented^{11, 14, 15}, although the effects of ECM components on osteogenic transformation of VICs have not been previously described. Our goals in these

experiments were two-fold: to identify ECM components that are appropriate for use in biomaterial scaffolds for tissue-engineered valves, and to gain a better understanding of the roles of ECM components found in native valves in valve calcification.

The findings presented in this manuscript are significant, and point to a vital role of ECM proteins in the regulation of VIC dysfunction, in addition to identifying important biomolecule candidates for inclusion in biomaterial scaffolds for the engineering of healthy valve tissue. It is certainly striking that even treatment with potent factors known to induce calcification could not induce VICs to form calcific nodules on two of the ECM proteins – fibronectin and collagen. This result implies that collagen and fibronectin actively perform functions to inhibit VIC calcification. These findings are also notable in their direct contrast to the vascular calcification literature, where collagen I and fibronectin have been found to promote the calcification of vascular smooth muscle cells *in vitro* [ref]. In this previous study, a calcifying subpopulation of vascular SMCs exhibited increased nodule formation and alkaline phosphatase expression when cultured on collagen I and fibronectin, which is the opposite of the trends observed with VICs on these same matrices.

There are numerous possibilities for the apparent calcification inhibition properties of collagen and fibronectin in VIC cultures. The effect could be integrin-specific, wherein integrin interactions with certain collagen or fibronectin adhesion peptides stimulate signaling pathways that block VIC activation to a myofibroblast phenotype. Integrin binding-stimulated pathways could also participate in the regulation of growth factor production, thereby altering the endogenous production of pro-calcific (TGF- β 1, BMPs) or anti-calcific (noggin, chordin) compounds. Alternatively, the difference between the ECMs could be much more passive in nature, with the ECM proteins sequestering or binding various cytokines or growth factors. It is known that TGF- β 1 can be sequestered by fibronectin, and indirectly by collagen (via decorin)^{25, 26}. Such binding could have the effect of either making the TGF- β 1 more available to the cells by acting as a local storage, activation, and delivery depot, or, conversely, it could make the growth factors less available to the cells by limiting their diffusion to the cells. If TGF- β 1 sequestration were playing a role in our system, we predict that the latter case would be more likely based upon our results, since the smallest degree of calcification was observed on the proteins known to sequester TGF- β 1. The identification of these potential mechanisms for ECM regulation of VIC calcification is the next step for this research, and will undoubtedly prove useful for better understanding valvular disease and developing potential treatments.

Meanwhile, the apparent inductive mineralization effects of fibrin, laminin, and heparin also have several potential explanations. In the cases of laminin and fibrin, the increased calcification of VIC cultures is consistent with studies performed on the mineralization of vascular smooth muscle cells. Specifically, other groups have documented that activation of the elastin-laminin receptor (ELR) in vascular smooth muscle cells increases the production of osteogenic markers²⁷, while inhibition of the ELR significantly reduces matrix mineralization²⁸. Fibrin is frequently associated with the generation of mineralized atherosclerotic plaques, and numerous bone matrix proteins, including osteocalcin and osteonectin, are co-localized with (and possibly bound to) fibrin in vascular calcification²⁹. The effect of heparin on VIC calcification, however, does not follow predictions made from

studies of vascular SMCs. The current work shows increased calcification of VIC cultures grown upon heparin coatings, and this is consistent with the data by Cushing et al. showing increased VIC α -SMA expression with heparin treatment²⁴. Treatment of vascular SMCs with heparin, on the other hand, results in inhibition of osteoblast markers and calcification³⁰. This difference further underscores the importance of performing even simple studies of VIC calcification and not assuming a direct extrapolation from the extensive SMC mineralization literature.

The current results for nodule count, size, total calcified area, and previous results regarding α -SMA expression²⁴ also raise an interesting question regarding which characteristics are most important in analyzing VIC dysfunction. It is not yet known which type of mineralization data – nodule size, nodule number, or total calcification area – is the most relevant for investigating valve dysfunction. However, in our experiments, we found that the expression of the mineralization marker alkaline phosphatase and the osteoblast differentiation transcription factor CBFA1, correlated well with calcific nodule number. This correlation does imply that nodule number may be a more accurate predictor of VIC dysfunction than nodule size or total calcified area. As mentioned earlier, correlating α -SMA expression with culture calcification is complicated, as not all α -SMA-positive VIC cultures will calcify. In fact, a recent publication shows that α -SMA levels of VICs on fibronectin are elevated above those on TCPS²⁴, yet, in our work, fibronectin was demonstrated to be one of the least supportive surfaces for calcification. Thus, it is feasible that elevated α -SMA can occur without leading to a pathological outcome (i.e. calcification). This difference emphasizes the importance of looking at ultimate pathological outcomes (i.e. calcification, expression of osteogenic markers) when evaluating VIC dysfunction and further sets the current work apart from a previous report on the expression of α -SMA by VICs cultured on select ECM coatings²⁴. Although α -SMA levels may not be able to predict calcification, it does appear that VICs must be activated to an α -SMA-positive myofibroblast phenotype in order to form calcific nodules. This statement is supported by our observation that all cultures with nodules stained positively for α -SMA, and that actin depolymerization studies resulted in significantly decreased nodule formation (data not shown).

It should be noted that the calcification observed here could possibly be altered by changing the mechanical environment of the culture conditions. In order to maintain constant substrate mechanics across all conditions, all of our studies were performed on cells cultured on TCPS-based surfaces. Culture on such a stiff substrate may, in itself, activate VICs to a myofibroblast phenotype³¹. In this sense, the absolute amount of calcification seen in the present study may have been amplified due to the stiff culture substrates. However, this hypothesis makes the results observed for collagen and fibronectin-coated surfaces even more striking. Because material mechanics undoubtedly play a role in regulating VIC calcification, it is important to keep in mind differences in substrate stiffness when comparing the current results to others published by investigators culturing VICs on soft gels^{7, 32}. However, these postulations do not change the trends observed between various ECM coatings in the present study, namely that, in a constant stiffness environment, VICs are significantly more likely to calcify in an environment containing laminin, heparin, or fibrin than they are when cultured upon collagen or fibronectin. The fact that the stiffness of

TCPS is not physiologically relevant for a normal valve environment does not change the fact that these different ECM components possess some property that is stimulating signals that lead to the induction (or inhibition) of myofibroblast and osteoblast-like characteristics.

There exists an obvious need for long-lasting valve replacements that are capable of growth and repair. Our findings demonstrate the crucial role played by the ECM in regulating VIC function, and we aim to apply these findings to the development of appropriate biomaterials for valve regeneration. ECM components are widely used either alone or in combination with other materials in the creation of biomimetic scaffolds for tissue engineering applications^{2, 32–37}. Past and current research with tissue-engineered valves provide powerful motivation for the creation of a bioactive environment for healthy valve formation³⁸. Discovery of ECM components that promote healthy VIC function may directly impact valvular biomaterial design; conversely, discovery of ECM components that stimulate a diseased phenotype may influence valvular biomaterial design with respect to what agents to exclude from the design. Thus, the conclusion from this work is that the incorporation of biological molecules to create biomimetic scaffold materials must be performed with caution regarding biomolecule selection. The ECM components investigated in this study were selected based upon either their presence in native heart valves³, or their use by other investigators designing scaffolds for tissue-engineered heart valves³². Our findings are exciting, as they confirm the promise for this research to lead to the rational design of appropriate biomimetic materials for valve tissue engineering, as well as a better understanding and treatment of valvular disease. Lastly, the expression of multiple mineralization markers within calcified cultures not only helps us to further characterize the factors involved in the valve calcification process, but also provides potential targets for future calcification inhibition experiments.

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Figure 1.

The average number of calcified nodules per well for VICs cultured on TCPS and ECM-coated conditions demonstrates the significant ECM-dependence of VIC calcification.

* $p < 0.05$ compared to corresponding TCPS condition; $n = 4$ wells per condition.

Figure 2.

Fold increase in average number of calcified nodules per well upon the addition of exogenous TGF- β 1, BMP-2, and BMP-7 to VICs cultured on 'anti-calcific' coatings (Collagen, FN) and a positive control (TCPS). * $p < 0.03$ compared to corresponding untreated control; $n = 3$ wells per condition. ANOVA

Figure 3.

Left Panels: Positive staining for α -SMA (green), a marker of VIC activation, is abundant in all calcified cultures, but sparse in non-calcifying cultures. Nuclei have been counterstained blue. *Right Panels:* Phase contrast images of representative calcific nodules (or absence thereof) in VIC cultures. Scale bar = 100 μ m.

A **B** **Figure 4.**

(a) Average size (μm²) of individual calcified nodules for VICs cultured on TCPS and ECM-coated conditions, and (b) the total calcified area (μm²) per well for VIC cultures. n=3 wells per condition, 2–44 nodules per well. *p<0.05 compared to TCPS.

Figure 5. SEM photomicrographs of calcified nodules on (a) TCPS, (b) laminin ($0.5 \mu\text{g}/\text{cm}^2$), and (c) fibrin ($1.5 \mu\text{g}/\text{cm}^2$). (d) The type of mineralization detected in VIC cultures on TCPS, represented as the number of nodules testing positive for each calcification type divided by the total number of nodules tested. Legend: DCPD = Dicalcium Phosphate Dihydrate, OCP = Octacalcium Phosphate, TCP = Tricalcium Phosphate, HAP = Hydroxyapatite. * $p < 0.02$ compared to DCPD; $n=4$ wells, 6 nodules/well.

Figure 6.

(a) Alkaline phosphatase (ALP) activity in VIC cultures varies with ECM coating, as does expression of CBFA1 (b). In (c), the overlay of ALP expression (empty squares), CBFA1 expression (solid circles), and nodule quantification (bars) on different ECM coatings illustrates the correlation between these separate observations. * $p < 0.05$ compared to TCPS; # $p < 0.05$ compared to lower concentration of same coating component; $n = 6$ wells per condition.