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Differences in Valvular and Vascular Cell Responses to Strain in Osteogenic Media

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Abstract

Calcification is the primary cause of failure of bioprosthetic and tissue-engineered vascular and valvular grafts. We used tissue-engineered collagen gels containing human aortic smooth muscle cells (HASMC) and human aortic valvular interstitial cells (HAVIC) as a model to investigate cell-mediated differences in early markers of calcification. The HASMCs and HAVICs were isolated from non-sclerotic human tissues. After 21 days of culture in either regular or osteogenic media with or without 10% cyclic strain at 1 Hz, the collagen gels were assessed for DNA content, collagen I, matrix metalloproteinase (MMP)-2 and glycosaminoglycan (GAG) content. The collagen gels containing HASMCs contained significantly greater amounts of collagen I and GAG compared to HAVICs. Although strain increased MMP-2 activity for both cell types, this trend was significant ($p \leq 0.05$) only for HAVICs. Cultured gels were also assessed for osteogenic markers calcium content, alkaline phosphatase (ALP), and Runx2 and were present at greater amounts in gels containing HASMCs than HAVICs. Calcium content, Runx2 expression, and ALP activity were also modulated by mechanical strain. The results indicate that cell-mediated differences exist between the vascular and valvular calcification processes. Further investigation is necessary for improved understanding and to detect biomarkers for early detection or prevention of these diseases.

Keywords

Calcification; tissue engineering; cyclic strain; aortic valve; collagen gels

1. Introduction

Cardiovascular disease is a major cause of mortality in the elderly population in the developed world. Vascular and valvular diseases affect approximately 25% of the over 65 year population [1–3], where calcification is manifested in both of these diseases. Calcification is also a major challenge and main cause of failure for tissue-engineered and bioprosthetic vascular grafts and heart valves [4]. However, unlike vascular calcification, relatively little is known about the cellular and molecular mechanisms of valvular

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calcification. Vascular and valvular calcification show similarities in risk factors suggesting a continuum of the same disease [5]. Both of these disease processes are now accepted to be an inflammatory condition [4,6] and are mediated by similar morphogens and growth factors. Vascular and valvular cells also demonstrate osteogenic differentiation during calcification [5,7]. Despite these similarities, how these diseases initiate and whether vascular or valvular cells are more susceptible to calcification is still not clearly understood.

Valvular cell behavior has commonly been related to that of vascular cells due to the proximity of these two physiological systems; however, recent studies suggest a more complex and unique behavior by valvular cells as compared to vascular cells [8,9]. Valvular endothelial cells show unique cell alignment and gene expression in response to shear stress as compared to vascular cells [10,11]. Similarly, vascular and valvular progenitor mesenchymal stem cells elicit phenotypic differences supporting unique behavior by these two cell types [12]. Valvular interstitial cells (VICs) are responsible for maintaining valve structure and function [13]. They also demonstrate a heterogeneous population in culture, whereas a subpopulation of these cells (activated VICs) show greater osteogenic behavior compared to others [14]. Therefore, VICs would be expected to elicit a more complex response to osteogenic environment than a homogeneous population of vascular smooth muscle cells. Likewise, differences have been observed between VICs and smooth muscle cells (SMCs) in 2D culture in spontaneous calcification, cell viability, and calcific nodule formation [12,15].

Mechanical forces are an important physiological component of the environment experienced by cells and likely contribute towards the calcification process. In the aortic valve, calcification predominantly occurs on the fibrosa side of the valve [16–18]. Since the two sides of the aortic valve experience differences in their mechanical environment, where the ventricularis sees predominantly uniform and the fibrosa encounters disturbed blood flow, it suggests that mechanical factors might contribute towards the side-dependent valve pathology. Similarly in blood vessels, calcification is observed in areas of major branches that experience lower hemodynamic forces [19–21].

In this study, we used tissue engineered collagen gels as a model to investigate events associated with vascular versus valvular calcification. Even though similarities exist in the calcification process between these two cell types, identifying differences might result in early detection and possible treatment options for either system. Since both cell types experience cyclic strain in their native environment, we also investigated the contributions of a representative physiological strain of 10% cyclic strain on calcification events for these two cell types. The collagen gels prepared for each cell type were divided in three groups: a) static gels cultured in base media (static ctrl), b) static gels cultured in osteogenic media (static ost), and c) gels cultured under 10% cyclic strain in osteogenic media (strain ost). Our research was based on the hypothesis that calcification is a process dependent on both the cell type and the mechanical environment experienced by the cells.

2. Materials and Methods

2.1. Primary cell isolation

Primary cell culture of HASMC and HAVIC were established from freshly obtained non-sclerotic tissues from heart transplant patients (one female, age 44 years, and three male, ages 22, 62, and 66 years) using a similar protocol as previously reported [8]. The study was approved by the institutional review board at Georgia Institute of Technology and Emory University and patient consent were obtained prior to surgery. The ascending aorta and the aortic valve tissues were obtained during transplant and were immediately processed for cell culture. Both the aorta (near the annulus) and the aortic valve leaflets were first minced

under sterile conditions and digested in type II collagenase solution in an incubated shaker for four hours. The resulting cell suspension was then filtered, centrifuged, and plated in T-75 flasks. Both types of cells were cultured in medium containing MCDB 131 (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Mediatech, Inc.), 1% penicillin-streptomycin (Mediatech, Inc.), 1% L-glutamine (Mediatech, Inc.), 0.0005 µg/ml FGF (PeproTech Inc., Rocky Hill, NJ), 0.01 µg/ml EGF (Invitrogen, Carlsbad, CA), 5 µg/ml Insulin (Invitrogen). Cells from four patients at passage 6 were used to prepare collagen gels.

2.2. Tubular collagen gel preparation and pneumatic bioreactor culture

A tubular mold for the collagen-cell mixture was created using a hollow glass rod, rubber stoppers, and a glass tube, as previously reported [22]. The mold also contained silicone sleeves inserted on the glass rods, where the surface of the sleeves was modified in 10N H₂SO₄ to promote adherence of collagen to the surface. The collagen gels were prepared using acid-soluble bovine dermal collagen type I (2 mg/ml, MP Biomedicals, Solon, OH) and either HASMC or HAVIC cells (1 million cells/ml) using an established protocol [23]. The collagen gels were cultured under static condition for 5 days before applying osteogenic conditions.

Cyclic distension was applied to the compacted collagen gels using a pneumatic bioreactor developed in our lab [22]. The base culture medium used for experimental conditions was same as previously mentioned culture medium without insulin. The osteogenic media consisted of dexamethasone (10 nM), β-glycerolphosphate (5 mM) and ascorbate-2-phosphate (50 µg/ml), all purchased from Sigma, St. Louis, MO [24]. As previously mentioned, the collagen gels prepared for each cell type were divided in three groups: a) static gels cultured in base media (static ctrl), b) static gels cultured in osteogenic media (static ost), and c) gels cultured under 10% cyclic strain at 1 Hz in osteogenic media (strain ost). Some preliminary studies were also performed where collagen gels were cultured under 10% strain in base media (strain ctrl). However, no significant difference was observed in the osteogenic markers in strain ctrl group as compared to static ctrl group and therefore was removed from the experimental groups. To determine the length of culture, we did preliminary studies, where the collagen gels were cultured for 9 or 21 days in the strain bioreactor. Since the trends for gene expression were similar for 9 versus 21 days but the osteogenic markers demonstrated stronger expression at 21 days, we selected 21 days as the culture duration for this study.

2.3. Hematoxylin & Eosin (H&E) and Von Kossa staining of tissue sections

The collagen gels were fixed in 10% formalin and processed using standard histology protocols. Paraffin embedded tissue sections (8 µm) were used for H&E and Von Kossa staining. For H&E, the sections were stained with Hematoxylin for 5 minutes and Eosin for 10 minutes using a Leica Autostainer. For Von Kossa staining, silver nitrate (5%) was added to the deparaffinized tissue sections and exposed to UV until the calcium salts turn black. The undissolved salts were then removed using 5% sodium thiosulfate, and the sections were counter-stained using nuclear-fast red.

2.4. Alkaline phosphatase (ALP) enzyme activity assay

ALP enzyme activity was measured using an assay as described by Osman et al. [24]. Briefly, the collagen gels were rinsed in calcium-free PBS and homogenized in RIPA buffer (Sigma). The ALP activity in the samples was measured against standard dilutions prepared from 10 mM p-nitrophenol (Sigma). 10 mM p-nitrophenyl phosphate (Fisher Scientific, Pittsburgh, PA) in substrate buffer (0.1 M glycine, 1 mM ZnCl₂, 1 mM MgCl₂, pH 10.4, all purchased from Sigma,) was then added to the samples and the resulting absorbance at 405

nm was measured at 0, 15, 30, 60, 90 and 120 minutes. The gradient (nmol/min) was then calculated for each sample and normalized to total protein content. The total protein content was determined using modified Lowry assay kit (Thermo Scientific, Rockford, IL).

2.5. Calcium content

The calcium content in the collagen gels was measured using a calcium specific Arsenazo dye reagent (Fisher Scientific). The collagen gels were first homogenized in 1N acetic acid and the calcium content was immediately determined using the dye. The absorbance of the reaction product was measured at 650 nm using a plate reader. The calcium content was then normalized to the wet weight of the gels.

2.6. Quantitative reverse transcriptase polymerase chain reaction (qPCR)

We isolated RNA from the collagen gels using RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA). cDNA synthesis was performed using SuperScript III First Strand kit (Invitrogen). The qPCR reaction were performed using SYBR green PCR master mix (Applied Biosystems, Foster city, CA) and a Step One Plus system (Applied Biosystems). The level of expression for each gene was quantified using known standards and normalized to reference gene QuantumRNA Classic II 18S Internal Standard (Applied Biosystems). The primers used were as follows: RunX2: Forward: GCACAAGTAAATCATTGAACTACAGAAA; Reverse: AGCCTGGCGATTAGAGTTTTG; Collagen I, alpha II: Forward: GCTACCCAACCTGCCTTCATG; Reverse: TTCTTGCA GTGGTAGGTGATGTTC.

2.7. DNA content

The DNA content in the collagen gels was measured using a DNA assay [23]. Briefly, the collagen gels were lyophilized overnight and rehydrated in 1 ml of buffer (50 mM Na₂HPO₄, 2 mM EDTA, 0.02% NaN₃, pH 7.4), then digested by adding 100 µl of Proteinase-K solution (10 mg/ml in ddH₂O, Invitrogen) and incubated at 60°C for 2 hours. The solubilized samples were fluorotagged with Hoechst 33258 dye (Sigma) and the resulting fluorescence was measured at 458 nm. Double stranded DNA from calf thymus (Sigma) was used as standard to calculate the mass of DNA present in each sample. The DNA content was normalized to the dry weight of each collagen gel.

2.8. Glycosaminoglycan (GAG) content

The GAG content was determined using dimethylmethylene blue (DMMB) dye (Polysciences Inc., Warrington, PA) on the homogenized samples used in the DNA assay. The resulting absorbance was measured at 525 nm. Chondroitin sulfate (Sigma) was used as a standard to determine total GAG content in each sample. The GAG content measured was normalized to the dry weight of the collagen gels.

2.9. Gelatin zymography

Gelatin zymography was used to determine matrix metalloproteinase (MMP) activity in the collagen gels. All of the products used for zymography were purchased from Invitrogen unless otherwise mentioned. Equal amounts (5 µg of protein) of non-reduced samples digested in RIPA buffer and MMP-2/9 ladder (Chemicon International) were loaded onto 10% zymogram gels. After electrophoresis, the gels were shaken in a renaturing buffer for 30 minutes and then cleared overnight in developing buffer at 37°C. The gels were then stained with colloidal blue staining kit to detect clear bands against a blue background. The band densities were calculated using gel analysis tool in Image J software.

2.10. Live/Dead assay

Live/Dead viability/cytotoxicity kit (Invitrogen) was used to detect cell viability in the collagen gels. Briefly, at the end of culture, the collagen gels were sliced in the radial direction to obtain thin rings. The sections were then incubated in Calcein AM and Ethidium homodimer-1 solution according to manufacturer's protocol. The collagen gel sections were imaged using a Zeiss LSM 510 confocal microscope.

2.11. Smooth muscle alpha actin (α SMA) staining

The paraffin-embedded tissue sections were deparaffinized and rehydrated following standard histology protocol. The tissue sections were blocked with 10% donkey serum (Sigma) and then incubated in mouse anti-human α SMA primary antibody (Sigma) for two hours at room temperature. The sections were then incubated in Alexa Fluor 488 donkey anti-mouse secondary antibody (Invitrogen) and Hoechst 33258 dye (Sigma) for an hour in dark, then mounted with mounting media, and imaged using a Zeiss LSM 510 confocal microscope.

2.12. Statistical analysis

Because cells isolated from four different patients were used in this study, fold changes were first calculated within the same patient to reduce patient-to-patient variability and then averaged for all patients. Three different comparisons were assessed: a) static ost normalized to respective static ctrl for either cell type, b) strain ost normalized to respective static ost for both cell types, and c) fold change in collagen gels containing HASMCs as compared to those containing HAVICs. Statistical significance was determined for the comparisons using 1-factor ANOVA. A p-value of ≤ 0.05 was considered significant.

3. Results

Greater matrix remodeling in collagen gels containing HASMCs than HAVICs

The collagen gels cultured in osteogenic media appeared more compacted in micrographs for both cell types, which also supports our visual observation. In some cases, the collagen gels prepared with HAVICs appeared less compacted than those with HASMCs. However, based on our visual observation from both preliminary and present studies, we found this difference to be patient dependent and not cell type dependent. Figure 1a shows H&E staining for the paraffin-embedded collagen gel sections. The sections cultured in static ost and strain ost condition stained brighter compared to static ctrls for both cell types. The collagen gels did not show any statistically significant difference in DNA content among culturing conditions or cell types (data not shown). The cell viability in the collagen gels cultured in regular or osteogenic media were also examined using live-dead assay. Most of the cells appeared alive (stained green) with few dead cells (stained red) for the collagen gels cultured in either culture media (data not shown).

Figure 2 shows the total GAG content in the collagen gels reported as fold changes for the various comparisons. Figure 2a demonstrates that the GAG content for the collagen gels cultured in static ost condition was greater ($p < 0.05$) for both HAVIC and HASMC cells when they were normalized to respective static ctrl gels; however, no significant increase for the GAG content was observed due to strain for the osteogenic condition (i.e. strain ost normalized to static ost) with either cell type. When comparisons were made between the cell types (i.e. HASMC/HAVIC), there was approximately 1.5 to two fold increase in GAG content in collagen gels containing HASMC cells for both static ost and strain ost groups when normalized to respective groups containing HAVICs (Fig. 2b, $p < 0.05$).

For collagen I mRNA expression, we observed a trend of lower collagen I mRNA expression for some experimental conditions when cultured in osteogenic media ($p=0.07$ for static ost normalized to static ctrl in HASMC, $p<0.05$ for strain ost normalized to static ost in HAVIC), but this trend was not consistent for the two cell types (Fig. 3a). Nevertheless, there was a significantly greater amount of collagen I mRNA in both static ost and strain ost groups in HASMCs than HAVICs for all of the patients (Fig. 3b, $p\leq 0.05$).

For matrix degradation, we observed an increase in MMP-2 activity for both static ost and strain ost conditions for both HASMCs and HAVICs (Fig. 4a). When the band intensities were measured and normalized to fold changes, we however did not find any significant difference in MMP-2 (pro and active) in static ost with respect to static ctrl for HAVICs, but a trend of greater amounts of active MMP-2 was observed for HASMCs (Fig. 4b, $p=0.09$). Strain decreased pro MMP-2 and increased active MMP-2 for both cell types. When a cell type comparison was made, we observed the same trend of increased active MMP-2 in HASMCs than HAVICs; however, only the static ost comparison between the two cell types was significant (Fig. 4c).

Elevated osteogenic marker expression in collagen gels containing HASMCs than HAVICs

Von Kossa staining on collagen gel sections demonstrated calcification under osteogenic condition for both cell types. We also observed more calcium deposition in collagen gel sections containing HASMCs than HAVICs for the same patient (Fig. 1b). The collagen gels showed an increase in total calcium content when cultured in osteogenic media under the static condition; however, this trend was significant for HASMCs only ($p<0.05$, Fig. 5a). Strain significantly increased calcium content for both cell types for osteogenic media. Similarly, there was significantly greater amount of calcium content in collagen gels containing HASMCs than HAVICs for both static ost and strain ost conditions (Fig. 5b). Figure 5c shows the actual calcium content normalized to wet weight to show the extent of calcification observed in the collagen gels for either cell type.

ALP enzyme activity significantly increased when the collagen gels were cultured in osteogenic media for both cell types (Fig. 6a, $p<0.05$). Application of strain under osteogenic condition reduced ALP activity for both cell types (Fig. 6b). There was also a significantly greater amount of ALP activity in HASMCs as compared to HAVICs for the same patient (Fig. 6c).

Runx2 mRNA expression was significantly increased in static ost gels when compared to respective controls for both cell types (Fig. 7a, $p<0.05$). Both cell types showed an increase in Runx2 expression under strain ost condition compared to the respective static ost condition; however, this trend was significant for HASMCs only. When comparisons were made between the two cell types, we observed a significant decrease in Runx2 expression in static ost, but a significant increase occurred only in the strain ost condition in collagen gels containing HASMCs (Fig. 7b).

α SMA mediated calcification for collagen gels containing either cell type

Immunohistochemistry staining demonstrated an increase in α SMA expression for both cell types in the collagen gels when they were cultured in osteogenic media (Fig. 8). The outer edges of the gels also commonly demonstrated stronger staining than the rest of the gels, possibly because that was the contact surface for the osteogenic media. Qualitatively, there were no apparent differences in α SMA staining in the gels due to strain for either cell type.

Discussion

This work investigated the contributions of cell type and 10% cyclic strain at 1 Hz on the events associated with calcification in vascular and valvular cells isolated from heart transplant patients using tissue-engineered collagen gels as a model. The main findings of this research have been that, within the same patient, collagen gels containing HASMCs demonstrated elevated amounts of ALP enzyme activity, calcium content, MMP-2, GAG content, and collagen I as compared to HAVICs in the osteogenic environment. Similarly, we observed strain modulated MMP-2, calcium content, and ALP activity for the osteogenic environment.

This study was limited by the fact that, while the cells were isolated from non-sclerotic tissue, they were not from healthy individuals. In addition, since the cells were isolated from neighboring locations, the mechanical environment experienced by the cell types would likely be more similar than HASMCs isolated from other locations in aorta. We therefore acknowledge that the differences observed in this study between HASMC and HAVIC might not be representative of all locations of the aorta due to the differences in mechanical forces observed by those cells. The contribution of endothelial cells on calcification events also was not investigated in this study.

Collagen gels have been successfully used to investigate calcification events in different physiological systems such as in bone [25], heart valve [26] and blood vessels [27]. A 3-D collagen gel provides an environment that mimics the native system better than 2-D and has been reported to promote an osteoblastic phenotype [25]. The tissue-engineered model thus provides a tool to determine factors involved in the initiation and progression of diseases *in vitro*. We observed that in our study, both cell types expressed osteogenic markers when cultured in osteogenic environment under static condition, supporting the use of collagen gel tissue engineered models for investigation of calcification events. HASMCs demonstrated significantly greater amounts of osteogenic markers in basic osteogenic media (without any procalcific growth factors) than HAVICs for the same patient. This trend was observed in all four patients investigated in this study and for both static ost and strain ost conditions. Further investigation is necessary to conclude that HASMCs are more predisposed to calcification; however, our results do demonstrate a significant difference in the level of response to osteogenic media between these two cell types. It may also be possible that the mechanical environment observed by valvular cells is more osteogenic than that observed by HASMCs. This suggests that the different response by HAVICs might possibly be a counter mechanism to function in the complex mechanical environment observed by the valves.

Calcified aortic valve diseases are usually accompanied by major extracellular matrix remodeling and tissue mineralization [4,14,28]. In our study, we also observed a greater matrix remodeling and compaction in the collagen gels cultured under osteogenic condition. Similar to the trend observed for osteogenic markers, HASMCs demonstrated greater amounts of matrix synthesis and remodeling (i.e. GAG, collagen I, and MMP-2) than HAVICs for the same patient.

Strain has been reported to promote osteogenic markers and matrix remodeling, such as calcium content [29,30] and MMP-2 synthesis [31], in various systems including cell-seeded collagen gels. Similarly, we observed strain enhanced calcium content for both cell types and Runx2 expression for HASMCs in our collagen gels. Likewise, greater MMP-2 expression has been reported in calcified valves and in atherosclerosis [32]. MMP-2 is strain dependent and is increased in both 2-D cell cultures and 3-D collagen gels in response to strain [31]. In our study, an increase in MMP-2, in particular active MMP-2, was observed for most patient cells for both osteogenic media and strain for either cell type. However,

when band intensities were calculated, some comparisons were not significant due to large patient-to-patient variability. We acknowledge that based on existing literature [31], it is possible that MMP-2 level might be influenced by strain alone. The greater amount of active MMP-2 in the collagen gels nonetheless is expected to have contributed towards the greater matrix remodeling and calcification observed under strain ost condition for both cell types.

ALP enzyme hydrolyzes phosphoesters and is an early marker for physiological mineralization [28]. While ALP is accepted to be upregulated during osteogenesis, its response to strain from *in vitro* cultures has been controversial [7,27,30,33–35] since its activity appears to be dependent on the time and strain regimen applied to the cells or 3-D matrices [33]. However, generally ALP activity has been reported to be down-regulated in response to similar levels of strain as applied in this study for a variety cell types [27,30,33]. In our study, we also observed that ALP enzyme activity was significantly increased under osteogenic environment with or without strain, but was lower in 10% strain samples for both cell types as compared to respective static ost condition. Since strain has been reported to downregulate ALP but increases calcium content [30] as observed in this study, strain likely has an elevated effect on some of the osteogenic markers than others. Additionally, since most of the matrix markers demonstrated increased expression due to strain, the increased calcification observed might be due to matrix remodeling rather than osteogenic differentiation. Further investigation is therefore needed to understand how different pathways might be influenced by strain in an osteogenic environment.

Calcification in diseased native tissue and *in vitro* studies have traditionally been reported to occur via apoptosis and α SMA expression. Calcification in cyclically stretched porcine valves has also been reported to be mediated by the TGF- β superfamily [36]. While the pathways and mechanisms involved are beyond the scope of this study, we tested if α SMA expression was enhanced in the collagen gels when they were subjected to an osteogenic environment. In fact, we observed that both cell types demonstrated stronger α SMA staining when cultured in osteogenic media as compared to static ctrl, suggesting α SMA/TGF- β -related pathways are likely altered in our collagen gels for osteogenic culture conditions; however, the influence of strain on α SMA staining was not apparent in this study. Further investigation would shed light on various osteogenic pathways that may have been activated for these two cell types for these culture conditions. Of particular interest would be the RANK [37], Notch1 [38], Sox9 [39] mediated pathways that has been recently reported to be involved in valvular calcification.

Conclusion

In this study we have demonstrated that for cells from the same patient, HASMCs express elevated levels of osteogenic markers and a greater matrix remodeling than HAVICs in response to an osteogenic environment. We also observed that strain modulates several of the osteogenic markers such as calcium content, Runx2, ALP enzyme activity for both cell types. A thorough understanding of the similarities and differences between vascular and valvular calcification could provide insight into the physiological basis of the disease and may ultimately lead to possible therapeutic approaches to prevent or treat calcification in either system. Likewise, our study emphasizes the importance of cell source in tissue engineering applications, and suggests that the investigation of different cell types is necessary to achieve desired tissue properties for *in vivo* applications.

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Figure 1.

H&E and Von kossa staining on paraffin embedded collagen gel sections: (a) H&E staining demonstrated brighter eosin staining for both osteogenic media and strain for both cell types. The collagen gels also appeared more compacted when cultured in osteogenic condition. (b) Von kossa staining show increased calcium deposition when they were cultured under osteogenic condition. Collagen gels prepared with HASMCs also demonstrated more calcium deposits than those prepared with HAVICs within the same patient. The images for each staining represent collagen gels prepared using cells from one patient. Image bar is 5 μm.

Figure 2.

Total GAG content in collagen gels containing either HASMC or HAVIC cells at 3 weeks: (a) Fold change in collagen gels cultured in static ost condition normalized to respective static ctrl and in strain ost condition normalized to respective static ost; (b) Fold change in collagen gels containing HASMCs normalized to those containing HAVICs. The data represents mean $\pm$ Std. error. * indicates $p \leq 0.05$. $n=4$ patients.

Figure 3.

Collagen I mRNA expression in collagen gels containing either HASMC or HAVIC cells at 3 weeks: (a) Fold change in collagen gels cultured in static ost condition normalized to respective static ctrl and in strain ost condition normalized to respective static ost; (b) Fold change in collagen gels containing HASMCs normalized to those containing HAVICs. The mRNA expression was normalized to million copies of 18S. The data represents mean $\pm$ Std. error. * indicates $p \leq 0.05$ and ^ indicates $p = 0.07$. $n = 4$ patients.

Figure 4. MMP-2 activity (inactive, active and total) was detected using gelatin zymography for collagen gel samples containing either HASMC or HAVIC cells at 3 weeks: (a) Representative gel images for HAVICs and HASMCs from same patient; (b) Fold change in collagen gels cultured in static ost condition normalized to respective static ctrl and in strain ost condition normalized to respective static ost; (c) Fold change in collagen gels containing HASMCs normalized to those containing HAVICs. The data represents mean $\pm$ Std. error. * indicates $p \leq 0.05$, ^ indicates $p = 0.07$, and # indicates $p = 0.09$. $n = 4$ patients.

Figure 5.

Total calcium content measured using calcium specific Arsenazo dye in collagen gels containing either HASMC or HAVIC cells at 3 weeks: (a) Fold change in collagen gels cultured in static ost condition normalized to respective static ctrl and in strain ost condition normalized to respective static ost; (b) Fold change in collagen gels containing HASMCs normalized to those containing HAVICs; (c) Total calcium content in collagen gels containing HAVICs and HASMCs normalized to wet weight. The data represents mean $\pm$ Std. error. * indicates $p \leq 0.05$. $n=4$ patients.

Figure 6.

ALP enzyme activity measured using an ALP assay for the collagen gels containing either HASMC or HAVIC cells at 3 weeks: (a) Fold change in collagen gels cultured in static ost condition normalized to respective static ctrl; (b) Fold change in collagen gels cultured in strain ost condition normalized to respective static ost; (c) Fold change in collagen gels containing HASMCs normalized to those containing HAVICs. The data represents mean $\pm$ Std. error. * indicates $p \leq 0.05$. $n=4$ patients.

Figure 7.

Runx2 mRNA expression in collagen gels containing either HASMC or HAVIC cells at 3 weeks: (a) Fold change in collagen gels cultured in static ost condition normalized to respective static ctrl and in strain ost condition normalized to respective static ost; (b) Fold change in collagen gels containing HASMCs normalized to those containing HAVICs. The mRNA expression was normalized to million copies of 18S. The data represents mean $\pm$ Std. error. * indicates $p \leq 0.05$. n=4 patients.

Figure 8. Immunohistochemistry for α SMA showed increased staining when the collagen gels containing either cell type were cultured in osteogenic media. The images represent collagen gel sections from one patient. Image magnification is 40X.