

Interstitial cells from left-sided heart valves display more calcification potential than right-sided ones: an in vitro study of porcine valves.

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Abstract

Calcification of cardiac valves is more frequently observed on the left-sided valves (aortic or mitral) than the right-sided ones (pulmonic or tricuspid). The causes for this preferential left-sided calcification remain relatively unknown. In this study we evaluated the capacity to calcify of interstitial cells in culture isolated from each of the four cardiac valves from healthy adult pigs. Interstitial cells were isolated from the leaflets of each 4 valves from three healthy young adult pigs and cultured in DMEM/FBS 10% in the presence or not of osteogenic additives (PCA) (ascorbic acid, dexamethasone, β -glycerophosphate). Proliferation rate was similar for the cells from each four valves. After a longer period in culture (>10 days), cells from each valve spontaneously formed several calcification nodules. This process was accelerated in presence of PCA (4-7 days). Alkaline phosphatase (AP) activity was highest in cells originating from the aortic and mitral valves respectively and least in those from the pulmonic and tricuspid valves. Culture with the PCA increased the AP activity by at least 50% for each valve but relative AP activity between cells from each valve origin remained the same (aortic>mitral>pulmonic>tricuspid). Interestingly, matrix Gla-protein mRNA levels, an endogenous calcification inhibitor, followed an opposite trend of expression for each valve. Interstitial cells from porcine cardiac valves share similarities although their capacity to calcify is more evident in valves from the left side of the heart. Interstitial cells from the aortic valve are those who displayed the most potential for calcification.

Introduction

Calcific aortic valve stenosis is the most frequent heart valve disease in developed countries and the most common cause of heart valve replacement in the elderly⁽¹⁾.

Mitral valve annulus calcification is also very prevalent in this population.

Calcification of cardiac valve leaflets leading to stenosis is usually observed on the left side of the heart, mostly in the aortic valve ^(2;3). The reasons for this preferential left-sided calcification remain relatively unexplored.

Valvular interstitial cells (VICs) are the most abundant cells of heart valve leaflets and are responsible for maintaining the structural integrity of the valve ⁽⁴⁻⁶⁾. They are believed to be a mixed cellular population made mostly of fibroblasts but also of myofibroblasts and few smooth muscle cells⁽⁷⁾.

Each heart valve (aortic (A), mitral (M), pulmonic (P) and tricuspid (T)) opens and closes during an average lifetime over 3 billions times. These valves thus must withstand mechanical fatigue by various mechanisms. Moreover, left-side valves (AV and MV) are exposed to greater pressure regimens than their right-side counterparts. Venous and arterial blood composition is also different between the left and right heart compartments. Despite these different mechanical and biochemical stressors, some intrinsic endogenous differences in cell population phenotype might exist. This hypothesis has never been explored.

Consequently, we wanted to evaluate if VIC from each four heart valves had a similar or different intrinsic calcification potential. In order to do so, we purified IC from porcine valve leaflets of each heart valve and compared their response to a pro-calcifying stimulus.

Material and methods

Materials.

IC isolation and culture:

The hearts of 4-5 months old pigs were removed from the chests and carefully rinsed in a ice-cold Krebs solution at pH 7.4 (112 mM NaCl, 4.96 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄ • 7H₂O, 2.52 mM CaCl₂ • H₂O, 29.76 mM NaHCO₃, 10.42 mM C₆H₁₂O₆). Leaflets of the four valves of each heart were dissected and placed in a 1mg/ml type 1 collagenase (Worthington, Lakewood, NJ) solution at 37°C for 30 min in 50% Dulbecco's modified eagle medium (DMEM) (Invitrogen Canada, Burlington, ON) and 50% phosphate buffered saline (PBS), containing 1% antibiotic / antimycotic solution (Invitrogen Canada). The leaflets were then gently scraped to remove remaining endothelial cells. The leaflets were then cut in pieces of about 2 mm² and incubated in a 4.5 mg/ml type 1 collagenase solution at 37°C for 12 hours in 50% DMEM and 50% PBS as above. The released interstitial cells were centrifuged, the supernatant removed and the cell pellet was washed two times with PBS. The cells were then placed in complete DMEM (10% fetal bovine serum (Hyclone, Logan, UT), 1% antibiotic / antimycotic, 1% glutamaxTM (Invitrogen Canada)). Cells in this study were used between passages 2 and 5. Where mentioned, heart valve interstitial cells were also culture in a pro-calcifying medium consisting of DMEM/FBS 10% supplemented with ascorbic acid (50 µg/ml), β-glycerophosphate (10 mM) and dexamethasone

(10 nM) (all from Sigma, Oakville Ont, Canada) for pre-determined study times. Culture medium was changed every 48 hours.

Immunohistology and stainings

Cells were put in culture in 4-well LabTek chambers (Nalge Nunc Internationnal, Naperville, IL). At the end of the protocol, cells were rinsed in PBS and fixed in methanol for 15 min at -20°C and then rinsed again in PBS. Fixed cells were then incubated in PBS with 0.5% bovine serum albumin (PBS-BSA; Sigma) for 15 min at room temperature (RT) and then with a monoclonal antibody specific for the smooth muscle actin- α (1.5 μ g/ml in PBS-BSA; Sigma) for 3 hours at RT. Slides were then rinsed in PBS several times and incubated with an Alexa 488 coupled anti-mouse antibody (2 μ g/ml in PBS; Molecular Probes, Leiden, Neitherlands) for one hour at RT in the dark. Cell nuclei were then stained with Hoechst 33342 (0.1 μ g/ml in PBS). Several (at least 3) pictures of each slides were taken on a Nikon Eclipse E600 (Tokyo, Japan) epifluorescence microscope using a computerized image analysis system (Image-Pro Plus, Version 4.5, Media Cybernetics, Silver Springs, MD) at 200X magnification. Positive cells counting for smooth muscle actin labelling was assessed by an observer blinded for the origin of the cells and is expressed as a ratio of labelled cells on total number of cells. Trichrome-Masson or Alizarin S red (calcification) staining were used to assess the effects of the pro-calcifying medium on interstitial cell cultures.

Alkaline phosphatase activity

Cells were harvested from 6-well plates by trypsinization and rinsed in PBS. They were then re-suspended in 250µl of 1% Triton X-100 in saline and incubated for 30 min at RT. After centrifugation at 380 x g for 10 min, the supernatant was collected and stored at -20°C. The alkaline phosphatase assay was conducted in an Elisa plate where 100µl of 5mM of 4-nitrophenyl phosphate (Sigma) in water, 80µl of sample were added and 20µl of AMP-MgCl₂ pH 10.5 (adenosine monophosphate (AMP) 0.5M, MgCl₂ 5mM). After a one-hour incubation at 37°C, the enzymatic reaction was stopped with 50µl of NaOH 0.6M and read on a plate reader at 405 nm. 4-nitrophenol (Sigma) diluted in NaOH 0.25M was used as a standard. In parallel, total protein content of the cell supernatant was determined using the Lowry method and results are expressed as the amount of 4-nitrophenol produced indexed for the total protein content of the sample tested.

Semi-quantitative RT-PCR

Total RNA extraction from the interstitial cell culture was done following the Trizol method. Reverse transcription and DNA amplification by PCR of the matrix Gla protein (MGP) and the Bone morphogenic protein-2 (BMP-2) as well as data analysis were carried out as described previously (^{8;9}) using the following primer pairs (Table 1).

Statistical analysis:

Results are presented as mean ± SEM unless specified otherwise. Paired Student t tests were used to compare the results between control and treated cells. One-

way analysis of variance was performed to compare serial data. Statistical significance was set at a p value of 0.05 or less using post-hoc Tukey's test. Data and statistical analysis were performed using GraphPad Prism version 4.02 for Windows, GraphPad Software (San Diego California USA).

Results

The isolated and purified interstitial cells from each heart valve had a clear fibroblastic morphology (see Figure 2) and did not stain for two endothelial markers namely CD31 and von Willebrand factor (not shown). Cells from the aortic valve showed a greater propensity to adhere to plastic after 24 hours in culture than their counterparts isolated from the other three heart valves (Figure 1, top). The proliferation rate was similar between interstitial cells from each valve (figure 1, bottom).

When let to grow in culture for 2 weeks, we observed the spontaneous formation of nodules staining positively for calcium salts by von Kossa and Alizarin red methods suggesting that a calcification process had taken place (figure 2A, B and C). This was true for cells isolated from each heart valve. More than two thirds of the primary interstitial cells from each valve stained strongly for the myofibroblastic marker smooth muscle α -actin and most of the remaining cells also showed some labelling (Figure 2D).

Valvular interstitial cells were cultured in presence of a osteogenic medium composed of β -glycerophosphate (BGP), ascorbic acid and dexamethasone for a period up to one week. As illustrated in Figure 2E, BGP had little effect on interstitial cells culture except for a non-significant trend for more calcification nodules after a 4 days of culture. The addition of ascorbic acid to the culture medium (figure 2 F) had a clear proliferative effect while dexamethosone induced cellular migration and increased proliferation (figure 2G). Put together, these three

agents rapidly induced calcification nodules formation (figure 2H). These observations were similar for cells purified from each heart valve.

In figure 3 are illustrated the effects of this osteogenic medium on interstitial cells isolated from each porcine heart valve. We tested the enzymatic activity of alkaline phosphatase which is a central player in the calcification process. As illustrated at day 4 and 7 in the normal culture medium, we observed that alkaline phosphatase activity was higher in the aortic valve cells followed respectively by the ones from the mitral, the pulmonic and finally the tricuspid valve. As expected, the addition of the osteogenic medium increased the alkaline phosphatase activity in cells from each valve but again, the alkaline phosphatase activity was still higher in cells from left heart valves.

Matrix Gla protein (MGP) is an endogenous inhibitor of calcification. We found that the expression of the messenger RNA encoding the gene of MGP was this time lower in interstitial cells isolated from the left heart valves and the highest in the tricuspid valve cells (figure 4). Osteogenic medium inhibited the expression of MGP mRNA in cells from each heart valve but this inhibition was strongest in the tricuspid valve.

The opposite response was observed for the expression of the osteopontin gene. This gene expression was higher in right heart valve interstitial cells but this time the osteogenic medium increased its mRNA levels with a trend for lower expression in left heart valves (figure 5). We did not observe any regulation of the

mRNA encoding for bone morphogenic protein 2 (BMP-2) in our cells either in the presence or not of an osteogenic stress (**figure 6**).

Discussion

We report that valvular interstitial cells isolated from porcine heart valves share several similarities as expected but also differences regarding their capacity to enter into a calcification process.

Although it is clear in the clinical context, that heart valves from the left chambers are more prone to undergo pathological calcification than their homologues from the right heart, the reasons for this have not been thoroughly studied. Intuitively, the higher pressure regimen and consequent mechanical stress in the left-side could be incriminated as a main factor for this difference. Different blood pH and oxygen contents could also be proposed as explanations for this phenomenon.

We observed in the current study of interstitial cells purified from young normal male pigs that several differences exist at least regarding the level of alkaline phosphatase enzymatic activity as well as for the gene expression of several players known for their implication in the pathological calcification process.

Alkaline phosphatase (AP) is central in the calcification of valvular interstitial cells *in vitro* ^(10;11). Interestingly, we observed that basal alkaline phosphatase enzymatic activity was higher in interstitial cells isolated from the aortic and mitral valves compared to the pulmonic and tricuspid valves. There was also a tendency for higher AP activity in semi-lunar valves (aortic and pulmonic) compared to the

atrio-ventricular ones. The opposite response was observed for the gene expression of the MGP (an endogenous inhibitor of calcification): right heart valves interstitial cells showed higher mRNA levels than those isolated from the aortic and mitral valve leaflets. This nice and opposite response for these markers associated with calcification and their negative controls respectively suggests that interstitial cells from each heart valve constitute per se either a different population or that they have developed specific adaptations and phenotypes according to their milieu (left or right heart circulation). It is difficult to obtain a definitive answer to this question, however. We did not observe any real differences in the phenotype of our four populations of interstitial cells in culture as for their proliferation capacity or their expression of smooth muscle cell actin which is a marker of myofibroblastic differentiation. This differentiation in culture of interstitial cells from a fibroblastic phenotype to a myofibroblastic one has been described before (¹²⁻¹⁵).

Osteopontin is another important marker often present in aortic stenosis lesions in patients(^{16;17}) and we found higher levels of expression of this protein in the right heart valves interstitial cells than in those isolated from the aortic and the mitral valves. This may look surprising at first glance. However osteopontin is also (¹⁸) known as a calcification inhibitor in vascular smooth muscle cells. It is thus not impossible that the right heart valves maintain a higher level of gene expression of this non-collagenous adhesion protein.

The response of valvular interstitial cells to an osteogenic stress caused by the addition of a phosphate source (β -glycerophosphate) as well as ascorbic acid and dexamethasone (^{19;20}) was as expected with a rapid formation of calcification nodules in culture. The activity of both AP and osteopontin gene expression increased when cells were exposed to this medium while the mRNA levels of MGP decreased. Similar observations have been done before but we observed in the current protocol that in most cases, the level of change was related to the basal levels of the studied factor. For example, AP activity was still higher in the interstitial cells from the aortic and mitral valves while MGP and osteopontin mRNA levels tended to be higher in cells from the pulmonic and tricuspid valves.

We also evaluated the separate action of each component of the osteogenic medium. Both glycerophosphate and ascorbic acid had apparently minor effects on the interstitial cell culture after 4 days. On the other hand, dexamethasone had dramatic effects on cell migration leading to the formation of cell clumps. Our observations here with valvular cells are not so different from the situation reported for marrow-derived mesenchymal stem cells more than a decade ago when this osteogenic medium was developed (²¹).

Surprisingly we did not observe any regulation of the expression of BMP-2 in our cells suggesting that the main source of this osteogenic factor in the calcification process taking place in aortic stenosis is not interstitial cells. Exogenous BMP-2

has been shown to induce bone formation in interstitial cells but here we failed to see any modulation of this factor in our cells (19).

Still, it is not clear why such a difference is present between the valvular interstitial cells from each heart valve in regard of calcification. A difference in terms of the capacity to contract collagen had been described recently between interstitial cells for the pulmonic and the aortic valve leading to the hypothesis for the explanation for increased aortic valve stiffness(22). We also have to keep in mind that our observations were made in absence of the natural barrier between these cells and the circulation i.e. the endothelial cells. How these cells mediate differences between the left and right heart sides of circulation to the interstitial cells has not been studied so far.

In conclusion, we report that interstitial cells from the aortic and mitral valves display a phenotype more prone to calcification which may in part provide some clues as to why calcification is more prevalent on valves from the left heart. It is not clear however if the source of this difference is related to a response to the environment of the valves.

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Gene	Oligonucleotide sequence	Amplicon size (bp)	Annealing (°C)
GAPDH	3'-CATATTTGGCAGGTTTCTCCAGACG 5'-TGACCCCTTCATTGACCTCCACTAC	381	60
MGP	3'-CGTGAAGCCACTGCACGAGAC 5'-GTCTGTGTCTATATAGTTCAG	448	55
BMP-2	3'-ATCCCCACGGAGGAGTTTATC 5'-CAACCTGGTGTCCAAAAGTCT	174	55
Osteopontin	3'- TGTCTTCTTCGCTCTTAGAGTC 5'- ACGACGCTGACCGATCC	500	59

Figure legends

Figure 1. Porcine valvular interstitial cells primary culture. Adhesion assay Top: Cells were seeded in 6-well culture plate (125000 cells per well) for the indicated before trypsinisation. Viable cell number was then assessed using a hemocytometer in presence of Trypan blue. This assay was made with six different replicates. Results are expressed as the number of cells/well \pm SEM. AV: aortic valve interstitial cells; MV: mitral valve; PV pulmonic valve and TV tricuspid valve. *: $p < 0.05$ vs. all other cell type. Proliferation assay Bottom: Cells were seeded in 6-well culture plate (125000 cells per well) for 24 hours ($n=12$ well/cell type). The culture medium was changed to remove floating cells. For half of the wells ($T=0$), cells were then trypsinised and counted while the other half was let to grow for 72 hours with a culture medium change after 48 hours. At the end, cell number was evaluated as above. Results are expressed as the fold change in cell number/well \pm SEM compared to $T=0$.

Figure 2. Formation of calcification nodules in aortic valve interstitial primary cell culture. (A, B, C) Cells were let to grow for 7 (A) to 14 (B,C) until spontaneous formation of calcification nodules which were stained using the Von Kossa method (B) or Alizarin S red (C). A majority of aortic valve interstitial cells in culture stained strongly for the α -smooth muscle actin (D). In presence of an osteogenic medium (ascorbic acid (E), β -glycerophosphate (F), dexamethosone (G)), aortic valve interstitial cells formed rapidly (4 days) calcification nodules. Panels E to H display

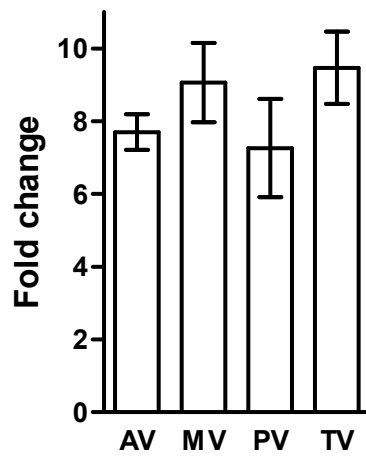
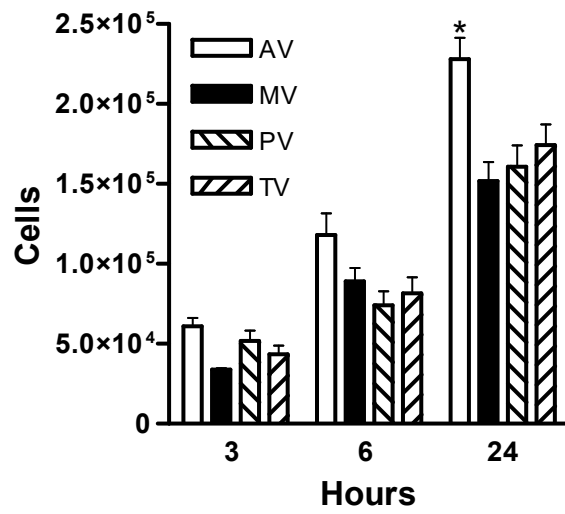
the effects of each components of this culture medium independently and in combination.

Figure 3. Alkaline phosphatase activity in porcine valvular interstitial cells in presence or not of an osteogenic medium after 4 and 7 days of culture. Cells were seeded (200000 cells/well) as described above and non-adhering cells were removed 24 hours later and osteogenic medium was added. Cells were then cultured for the indicated periods of time before trypsinisation and enzymatic assay as described in the Material and methods section. Alkaline phosphatase activity is expressed as nmoles of 4-nitrophenol formed/mg protein/min of 6 different replicates. AV: aortic valve interstitial cells; MV: mitral valve; PV pulmonic valve and TV tricuspid valve. *: $p < 0.05$ vs. AV and ¶: $p < 0.05$ vs. corresponding cell type in control (Ctrl) culture medium.

Figure 4 Matrix Gla protein mRNA levels in porcine valvular interstitial cells in presence or not of an osteogenic medium after 7 days of culture. Cells were seeded (125000 cells/well) as described above and non-adhering cells were removed 24 hours later and osteogenic medium was added. Semi-quantitative evaluation of mRNA levels was performed as described in the Material and methods section. Results are expressed as arbitrary units (AU) \pm SEM of six different replicates. AV: aortic valve interstitial cells; MV: mitral valve; PV pulmonic valve and TV tricuspid valve. *: $p < 0.05$ vs. AV and ¶: $p < 0.05$ vs. corresponding cell type in control (Ctrl) culture medium.

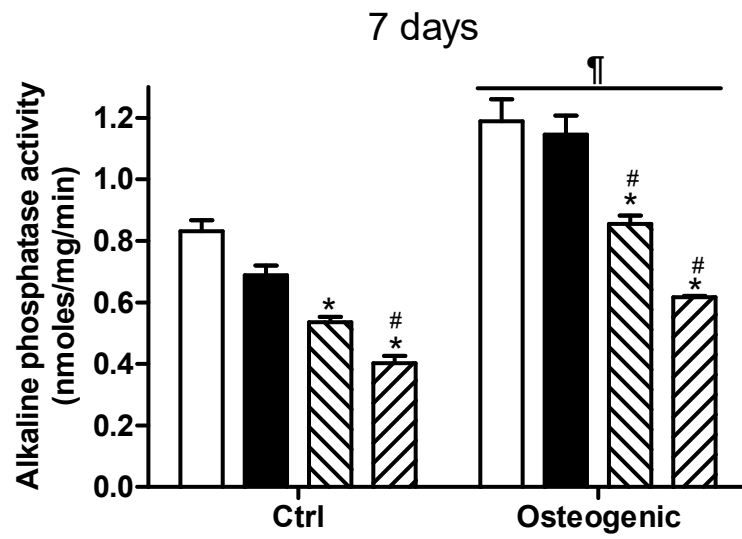
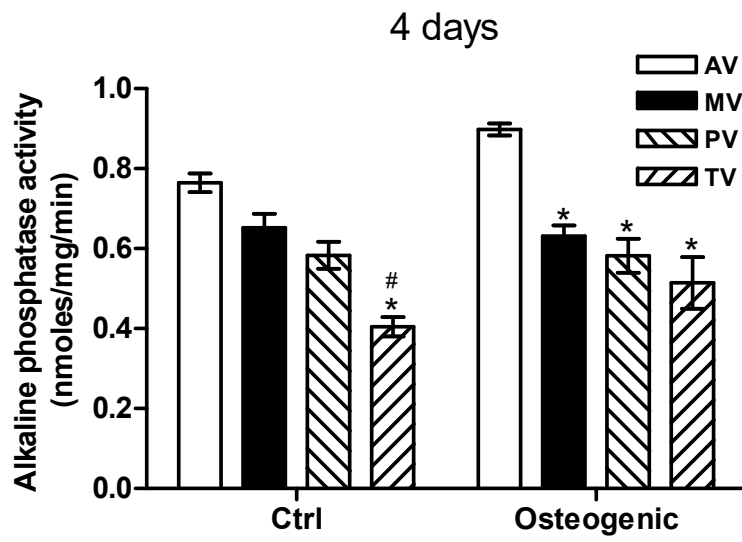
Figure 5. Osteopontin mRNA levels in porcine valvular interstitial cells in presence or not of an osteogenic medium after 7 days of culture. Cells were seeded (125000 cells/well) as described above and non-adhering cells were removed 24 hours later and osteogenic medium was added. Semi-quantitative evaluation of mRNA levels was performed as described in the Material and methods section. Results are expressed as arbitrary units (AU) \pm SEM of six different replicates. AV: aortic valve interstitial cells; MV: mitral valve; PV pulmonic valve and TV tricuspid valve. *: $p < 0.05$ vs. AV and ¶: $p < 0.05$ vs. corresponding cell type in control (Ctrl) culture medium.

Figure 6. Bone morphogenic protein-2 (BMP2) mRNA levels in porcine valvular interstitial cells in presence or not of an osteogenic medium after 7 days of culture. Cells were seeded (125000 cells/well) as described above and non-adhering cells were removed 24 hours later and osteogenic medium was added. Semi-quantitative evaluation of mRNA levels was performed as described in the Material and methods section. Results are expressed as arbitrary units (AU) \pm SEM of six different replicates. AV: aortic valve interstitial cells; MV: mitral valve; PV pulmonic valve and TV tricuspid valve.



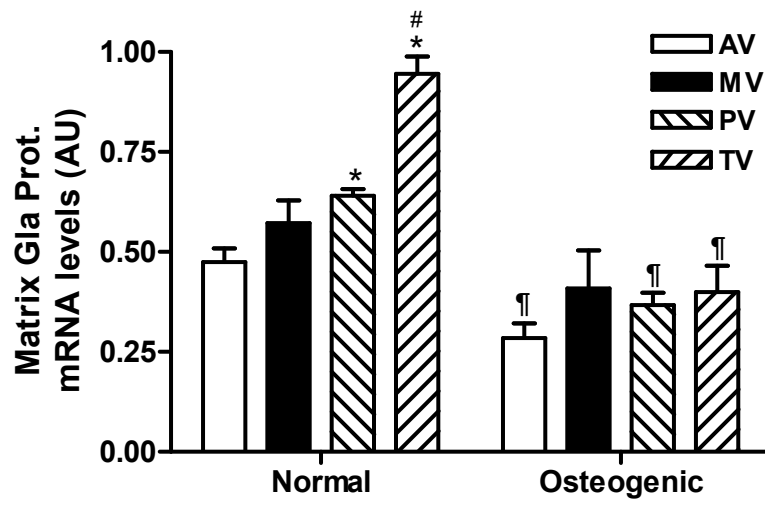
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Figure 2



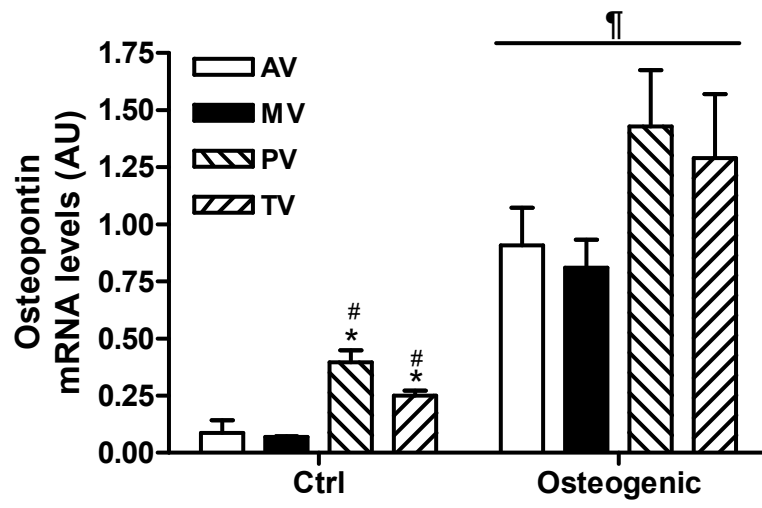
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Figure 3



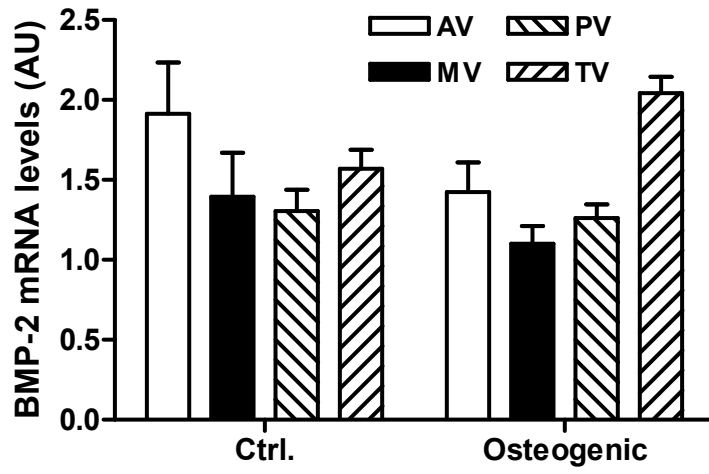
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Figure 4



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Figure 5



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Figure 6