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Co-culture induces alignment in engineered cardiac constructs via MMP-2 expression

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Abstract

Cardiac tissue engineering has been limited by the inability to recreate native myocardial structural features. We hypothesized that heart cell elongation and alignment in 3D engineered cardiac constructs would be enhanced by using physiologic ratios of cardiomyocytes (CM) and cardiac fibroblasts (CF) via matrix metalloproteinase (MMP)-dependent mechanisms. Co-cultured CM and CF constructs were compared to CM-enriched constructs using either basal media or media with a general MMP inhibitor for 8 days. Co-cultured constructs exhibited significantly increased cell alignment ($p<0.0002$), which was eliminated by MMP inhibition. Co-cultured constructs expressed substantial active MMP-2 protein that was not present in CM-enriched constructs, increased pro-MMP-2 ($p<0.001$), and reduced pro-MMP-9 ($p<0.001$) expression. Apoptosis was decreased by co-culture ($p<0.05$), independent of MMP inhibition. These results demonstrated that co-culture of CF in physiologic ratios within engineered cardiac constructs improved cell elongation and alignment via increased MMP-2 expression and activation, and also improved viability independent of MMP activity.

Keywords

Cardiac tissue engineering; matrix metalloproteinase; self-assembling peptide gel

Introduction

Cardiac fibroblasts (CF), which comprise roughly two thirds of the total adult heart cell population,[1,2] play a pivotal role in extracellular matrix (ECM) remodeling in cardiac tissue by regulating ECM synthesis and degradation through matrix metalloproteinase (MMP) expression.[3] Hence, CF may enhance myocardial regeneration by mediating ECM remodeling and cardiomyocyte (CM) migration, alignment, and elongation. Moreover, scar formation following myocardial infarction (MI) is associated with excessive ECM deposition by CF and altered MMP expression and activation.[4,5] Therefore a better mechanistic understanding of CF-CM interactions and how these interactions affect MMP-dependent remodeling could potentially enable positive cardiac remodeling and regeneration post-MI, while inhibiting scar formation.

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Tissue engineered cardiac constructs provide a model for mechanistic studies of cardiac remodeling and have the potential for enhancing myocardial repair in vivo.[6,7] To improve contractile function, many previous studies used CM-enriched (i.e. CF-depleted) cell populations to generate cardiac constructs.[8,9] However, recent studies showed superior properties for constructs with mixed heart cell populations as compared to CM-enriched populations.[10] We showed improved contractile function of constructs made by seeding CM-enriched heart cells in close proximity to pre-cultured CF by using Matrigel.[11] However, the resulting cardiac constructs displayed limited cell elongation and alignment, demonstrating the limited potential of CF in close proximity to induce CM elongation and alignment. Furthermore, mechanistic studies were problematic due to the presence of Matrigel, which is derived from the Engelbreth-Holm-Swarm mouse tumor and contains unknown quantities of ECM and growth factors.

For the present study, cells were embedded within a 3D self assembling peptide nanofiber (NF) gel to allow for mechanistic studies. The NF gel was comprised of the 16 amino acid sequence $(RADA)_4$, which is an ionic, self-complementary oligopeptide that gels upon contact with physiological ion concentrations.[12,13] Similar NF gels, when used as 2D substrates for cells cultured in monolayers, yielded improved survival and spatial reorganization of CM [14] and 3D capillary network formation by endothelial cells (EC).[15,16] Moreover, intramyocardial injection of NF gels in rodents promoted EC recruitment and, in conjunction with incorporated growth factors, enhanced the regeneration of ischemic myocardium.[17,18] Unlike Matrigel or naturally occurring hydrogels (e.g. collagen), NF gels do not contain ECM proteins, growth factors or cell derived molecules that could hinder in vitro mechanistic studies.

We hypothesized that cell elongation, alignment, and network formation in 3D engineered cardiac constructs could be enhanced by using co-cultured CF and CM in physiologic ratios to improve cell-cell networking via MMP-dependent mechanisms. To test this hypothesis, we compared co-cultured constructs with CM-enriched constructs in either the absence or presence of MMP inhibition. The results demonstrated that a physiologic ratio of CM to CF significantly enhanced MMP expression, cell alignment and viability, and also demonstrated that MMP-based ECM remodeling played a significant role in determining cardiac construct architecture.

Materials & Methods

Heart cells were obtained from 2-day-old neonatal Sprague-Dawley rats, as previously described.[19] Briefly, ventricles were isolated, minced, and incubated with 0.06% (w/v) trypsin overnight at 4°C, then digested at 37°C with agitation in 0.1% (w/v) type II collagenase (Worthington, Lakewood, NJ). The yield was 5 to 6 million cells per heart and viability was 90–95% by trypan blue exclusion. We recently demonstrated that these freshly dissociated ventricular cells had a CM:CF ratio equal to 0.67 [11]. Moreover, the subpopulation of these heart cells that remained unattached after 75 minutes of pre-plating in conventional culture dishes had a CM:CF ratio of 1.05, while cells that rapidly adhered and were passaged once had a CM:CF ratio of 0.24.[11]

3D constructs were prepared by embedding 3 million cells in 100 μL of self-assembling NF gel (Puramatrix™, 3DM, Cambridge, MA). Two groups of constructs were prepared: (i) a Co-culture group, made using a 1:1 mixture of CF and CM-enriched cells, and (ii) a CM-enriched group, made using a similar total number of cells that were CM-enriched. Prior to suspension in self-assembling peptide, cells were washed, centrifuged, and resuspended to a total volume of 50 μL in 20% sucrose solution. To each 50 μL of cell/sucrose mixture was added 50 μL of 1.0% (w/v) of self-assembling peptide. The resulting mixture was mixed thoroughly and pipetted into 12 mm culture inserts, (Millipore, Billerica, MA) within standard 24-well dishes containing ~1 mL of medium. NF gels were incubated for 15 minutes at room temperature,

followed by incubation for 45 minutes in a cell culture incubator to complete gelation. Gelled constructs were cultured free floating in 35-mm Petri dishes containing 5 mL of either basal medium or media supplemented with the general MMP inhibitor doxycycline at 200 mM. This inhibitor and concentration were selected based on a recent in vitro dosing study showing effective MMP inhibition with minimal effects on cell function or viability.[20] Basal media consisted of Dulbecco's modified Eagle medium containing 4.5 g/L glucose, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Media were completely replaced every 2 days. On day 8, constructs were bisected and either fixed for histology or stored at −80°C for protein isolation.

Specimens for histological analyses were fixed overnight in 10% neutral buffered formalin, dehydrated, paraffin embedded, and sectioned to a thickness of 5 μm. Immunohistochemical staining for cardiac troponin-I (Tn-I) was performed in triplicate as described previously.[19, 21] Briefly, sections were permeabilized, and incubated with monoclonal mouse anti-cardiac Tn-I (clone 23C6, Biodesign, Saco, ME) diluted 1:150 in PBS containing 0.5% Tween 20 and 1.5% horse serum for 1 hour at 37°C in a hydrated chamber. Sections were then incubated with secondary antibody and avidin-biotin complex (horse anti-mouse immunoglobulin G, Standard Elite ABC kit, Vector, Peterborough, UK) according to manufacturer's instructions. The antibody binding was visualized after incubation with 3,30-diaminobenzidine (Sigma-Aldrich, St Louis, MO) for 5–10 minutes and counterstaining with Harris hematoxylin (Sigma) using a Zeiss Axiovert (Carl Zeiss, Thornwood, NY) microscope at 1000X magnification.

Apoptosis was determined in 3 paraffin-embedded specimens per group using the deoxynucleotidyl transferase biotin 2-deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay (Roche, Indianapolis, IN) according to manufacturer's instructions. Stained sections were mounted on slides with DAPI-containing mounting media (Molecular Probes, Carlsbad, CA) to visualize cell nuclei. Total (DAPI-positive) and apoptotic (TUNEL-positive) cells were manually counted for 4 randomly selected regions for each test specimen. Apoptotic cells were expressed as a percentage of total cells.[19]

Confocal immunofluorescent staining for filamentous actin was determined for three formalin-fixed specimens per group. Briefly, specimens were washed 3X with PBS, incubated with 1% Tween for 20 minutes at room temperature, then incubated with Alexa Fluor 488-phalloidin for 1 hour at room temperature and mounted intact on slides. Intact constructs were visualized on a Zeiss LSM510-Meta confocal microscope at 250X (Whitehead Institute, Cambridge, MA). 3-D projections were created from the scanned layers through the thicknesses of immunostained sections. Cell alignment in these images was measured using the Fast Fourier Transform (FFT) in a custom-written Matlab routine (Engelmayr, unpublished), similar to previously described methods.[22] The orientation index equaled 1 minus the ratio of the minor to major axis lengths of the binary thresholded FFT image. Therefore, an orientation index value of 0 indicated completely random orientation, while a value of 1 indicated unidirectional cell alignment.

Amounts of pro- and active MMP-2 and MMP-9 were determined for four specimens per group using gelatin zymography from isolated protein as described previously.[23] Constructs were disrupted in CelLytic lysis buffer (Invitrogen, Carlsbad, CA) and total protein content was determined according to manufacturer's instructions (Biorad, Hercules, CA). Equal amounts of total protein (30 μg) were electrophoresed on each lane of 10% SDS gels containing 1% gelatin, then the protein was renatured, incubated overnight at 37°C, and stained with Coomassie blue to visualize enzymatic activity (all reagents and gels from Biorad). Enzymatic activity was visualized as clear bands against the blue stained gel. Bands were visualized, quantified and normalized by MMP-2/MMP-9 standards (Chemicon/Millipore, Billerica, MA).

Statistically significant differences between groups were assessed by 2-factor ANOVA in conjunction with Tukey's *post hoc* test using Statistica version 7 (StatSoft, Tulsa, OK).

Results

Cell alignment and cell-cell networking improved in co-cultured constructs

Confocal imaging of filamentous actin allowed assessment of cell-cell network formation and quantitative analysis of cell orientation (Fig. 1). Co-cultured constructs displayed large, multicellular networks comprised of elongated cells locally oriented in the same direction (Fig. 1A). CM-enriched constructs formed fewer and smaller networks comprised of less elongated cells (Fig. 1B), with 39% less cell alignment than co-cultured constructs ($p<0.0002$, Fig. 1E). MMP inhibition significantly decreased cell elongation, alignment, and interconnectivity in both co-cultured and CM-enriched constructs (Fig. 1C–E).

Cardiac troponin I expression confirms cardiomyocyte elongation in co-cultured constructs

Immunological evaluation of cardiac troponin I (Tn-I) confirmed the presence of CM and maintenance of cardiac phenotype in all experimental groups (Fig. 2). In co-cultured constructs, CM elongation was prevalent and large, aligned CM networks were observed throughout the samples (Fig. 2A). Some CM clustering was evident in the CM-enriched constructs however CM elongation was not commonly observed (Fig. 2B). MMP inhibition dramatically reduced cell-cell network formation and CM elongation and alignment in both co-cultured and CM-enriched constructs (Fig. 2C–D). The lack of cell elongation, clustering and orientation with MMP inhibition in both CM enriched and co-cultured constructs correlated well with the filamentous actin data. The presence, especially in co-cultured constructs, of Tn-I positive cells in elongated cell networks demonstrated that CM were prominently elongated and involved in aligned networks.

MMP expression and activation correlated with cell orientation and elongation

Quantification of MMP-2 protein demonstrated substantial expression of pro-MMP-2 protein in both co-cultured and CM-enriched constructs (Fig. 3). Pro-MMP-2 levels were significantly higher in co-cultured constructs than CM-enriched constructs ($p < 0.001$), independent of MMP inhibition. Active MMP-2 expression was substantial in co-cultured constructs, but was undetectable in CM-enriched constructs. In co-cultured constructs, MMP inhibition significantly decreased the amounts of pro-MMP-2 and active MMP-2 by 40% and 74%, respectively (Fig. 3A and 3B). In CM-enriched constructs, MMP inhibition significantly decreased the amount of pro-MMP-2 by 18% (Fig. 3A).

Quantification of MMP-9 protein demonstrated less overall expression than that of MMP-2 in all experimental groups (Fig 3C). Interestingly, the trend for pro-MMP-9 was opposite that of pro-MMP-2: pro-MMP-9 levels were significantly lower in co-cultured constructs than in CM-enriched constructs ($p<0.001$) independent of MMP inhibition. None of the samples tested displayed detectable levels of active MMP-9. In co-cultured and CM-enriched constructs, MMP inhibition significantly decreased the amounts of pro MMP-9 by 29 % and 22% (Fig. 3C).

Apoptosis was reduced by co-culture independent of MMP activity

Co-cultured constructs exhibited 53% less apoptosis than CM-enriched constructs ($p < 0.05$, Fig. 4), while apoptosis was not significantly increased by the MMP inhibitor doxycycline. Staining was stronger and more diffuse in CM-enriched constructs, whereas this staining was more punctate and highly localized in co-cultured constructs.

Discussion

Overall, this study identified significant beneficial roles for cardiac fibroblasts cultured within engineered cardiac constructs, including MMP-mediated increases in cell alignment, elongation, and network formation, and a non-MMP-mediated reduction in apoptosis.

The finding that co-cultured CF significantly improved cell elongation and alignment only if MMP expression was not blocked demonstrated that MMPs played a significant role in cardiac construct remodeling. CF co-culture increased MMP-2 expression and, in particular, MMP-2 activation, while reducing MMP-9 expression (Fig. 1–Fig. 3) in association with significantly increased cell alignment and clustering. MMP-2 and MMP-9 expression and activation are consistently required for cardiovascular and other cell types to migrate, organize and remodel ECM components, whereas reduced or dysregulated MMP activity can diminish cellular capability for remodeling.[4,20,24,25] Therefore one possible explanation for the suboptimal cell elongation and interconnection observed in the present and previous studies of CM-enriched (CF-depleted) constructs could be the minimal MMP-2 expression and activation. While all constructs in the present study contained pro-MMP-2 protein, active MMP-2 protein was detectable only in co-cultured constructs (Fig. 3B). Surprisingly, while increased MMP-2 expression often leads to increased MMP-9 expression,[23–26] in the present study this did not occur (Fig. 3), suggesting further nuclear signaling events may be occurring to suppress MMP-9 expression.

Native myocardial tissue is highly aligned and engineered cardiac constructs aim to recreate this cellular alignment to optimize function and maintain CM phenotype.[6] We[21,27] and others[6,7,28] previously showed that mechanical or electrical stimulation can improve cell alignment and collagen deposition in cardiac constructs. In the present report, we demonstrated that significant cell alignment and multi-cellular network formation can be achieved solely through modulation of the ratio of CF to CM cells, independent of physical stimulation. Cells within cardiac constructs were aligned by biological cues provided by co-culturing CF in physiologic ratios within engineered cardiac constructs, which activated MMP-2 and other signaling molecules. Further studies are warranted to test whether physical stimulation of co-cultured CF and CM constructs can further improve CM elongation and alignment.

We demonstrated that co-cultured CF significantly reduced apoptosis in cardiac constructs in a manner that was independent of MMP-mediated structural changes. These data give further evidence to an emerging hypothesis referred to as the ‘paracrine effect’,[29] wherein cells, including fibroblasts, benefit injured CM by the secretion of growth factors, cytokines, and other signaling molecules. In particular, both human and rodent studies have demonstrated that transplantation of supporting cells (e.g. fibroblasts, smooth muscle cells) enhanced function of injured myocardium.[30,31] Consistently, in previous in vitro studies of 3D cardiac constructs, we showed significant improvements in contractile function through culturing CF in close proximity to CM,[11] and significant reductions in apoptosis by providing supplemental growth factors.[19] Together, these findings suggest that the CF-mediated ‘paracrine effect’ in our co-cultured constructs significantly affected cell viability but not elongation or alignment, whereas MMP-mediated remodeling had little or no effect on viability.

The present study utilized a self-assembling peptide NF gel for embedding and culturing heart cells in 3D constructs. A distinct advantage of the NF gel compared to previously validated hydrogels (Matrigel, collagen gel), was that the NF gel maintained its mechanical integrity throughout 8-days of high density co-culture without exogenous ECM or growth factors, thereby providing a good 3D model for mechanistic studies of CM-CF interactions and construct remodeling. The performance of NF gel was comparable to Matrigel[19] with respect

to cell viability of embedded heart cells after 8 days of static culture. However, one disadvantage of the NF gel compared to previously validated hydrogels was in regard to development of macroscopic construct contractility. In particular, although localized beating of individual and small groups of CM within constructs based was consistently observed, macroscopic contractions were not. We examined whether lower NF gel concentrations would yield contractile constructs, based on previous reports that NF gel concentration was directly related to stiffness[32] and that 2D cell culture substrates with Young's modulus of ~12 kPa yielded optimal differentiation of C2C12 myoblasts.[33] However, constructs made using 0.2% and 0.5 % (w/v) NF gel displayed similar contractility properties, and concentrations lower than 0.2% would not gel. Of note, the estimated shear modulus (G') of the tested 0.5% and 0.2% (w/v) NF gels were respectively ~ 2.6 kPa and 0.2 kPa [32] and as such were orders of magnitude stiffer than Matrigel for which $0.03 < G' < 0.05$ kPa.[34]

In summary, the present study demonstrated some of the positive effects that CF can impart on the structural organization and survival of CM in 3D cardiac constructs, and also identified some of the underlying molecular mechanisms. The results demonstrate that CM elongation and alignment can be achieved by utilizing a physiologic ratio of CM and CF to promote MMP-2 mediated remodeling. With better understanding and control of the mechanisms underlying CF mediation of in vitro cardiac tissue formation, including increased MMP-2 production and activation, it may be possible to create more effective strategies for cardiac repair and regeneration while also gaining valuable information on the regulation of CM function.

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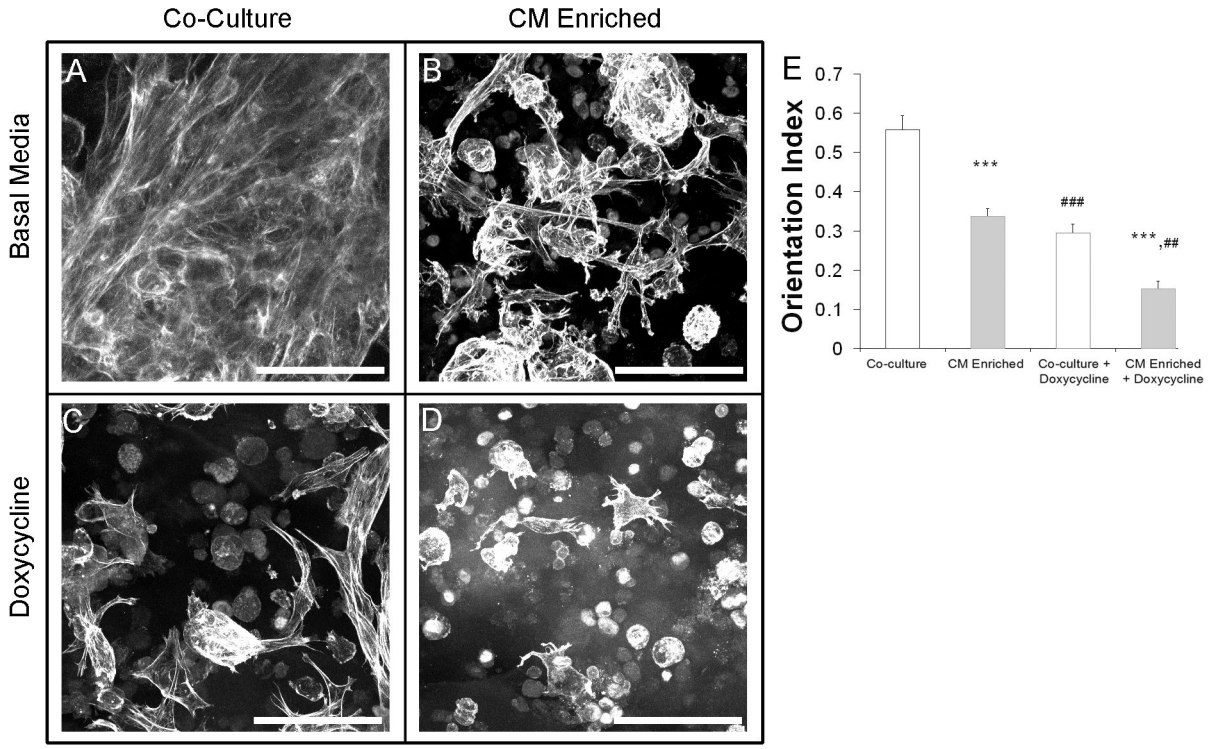

Figure 1.

Confocal imaging of filamentous actin in 8-day constructs. Representative images of Co-cultured (A,C) and CM-enriched (B,D) constructs cultured in basal media (A,B) and in the presence of a MMP inhibitor (C,D). Scale bars: 50 μm. (E) Orientation index (arbitrary units, higher values indicate a higher degree of orientation to a maximum of 1). Data are the mean ± SEM of triplicate samples. Two symbols (**,##) indicate $p<0.001$; three symbols indicate $p<0.0002$.

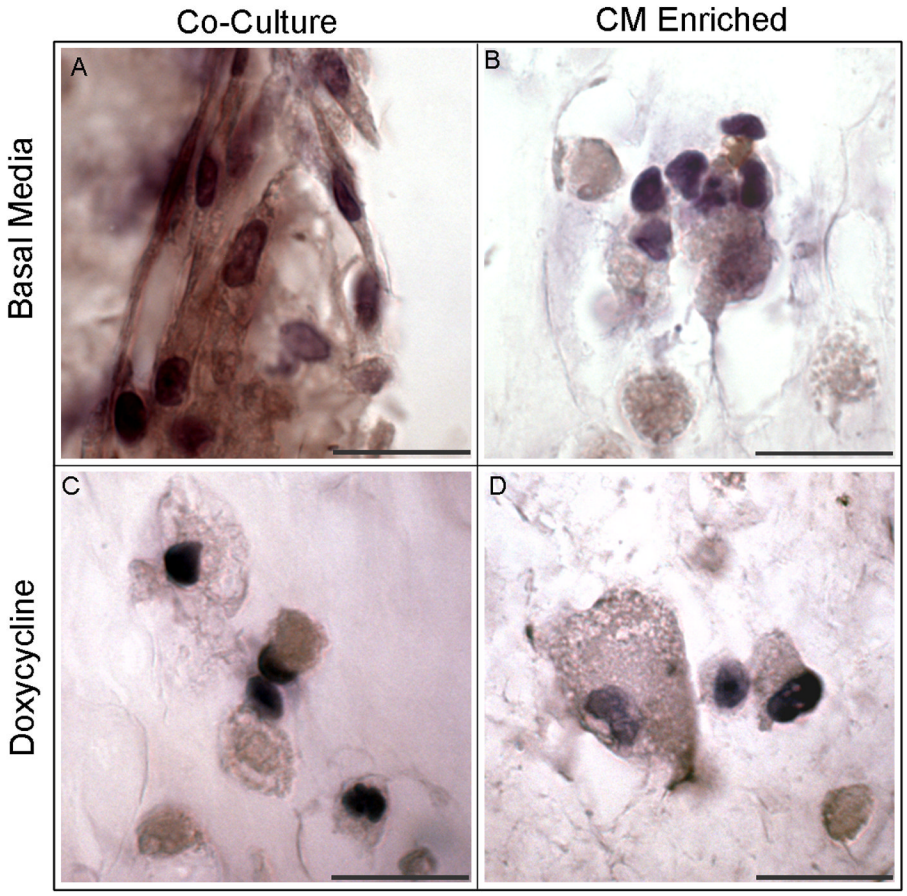

Figure 2.
Immunohistochemical staining for cardiac troponin-I in 8-day constructs. Representative images of Co-cultured (A,C) and CM-enriched (B,D) constructs cultured in basal media (A,B) and in the presence of a MMP inhibitor (C,D). Scale bars: 20 μm.

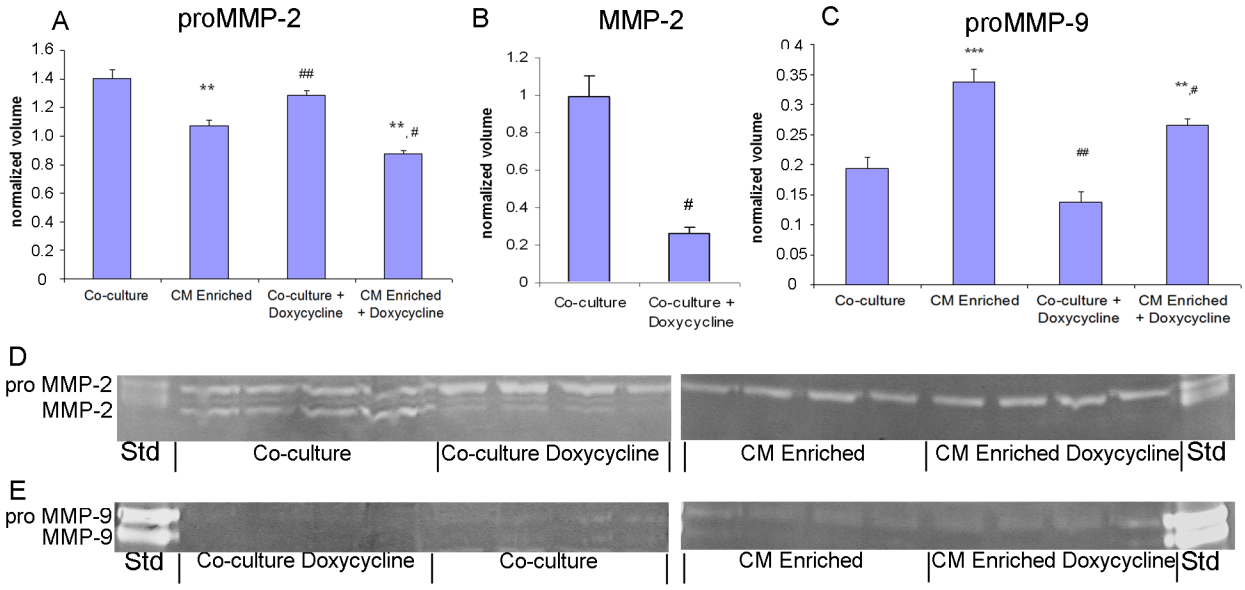

Figure 3.

Matrix metalloproteinase expression in 8-day constructs. Quantification of proMMP-2 (A), active MMP-2 (B), and proMMP-9 (C) proteins based on gelatin zymography images (D,E). Data are the mean ± SEM of n=4 samples. One symbol (*, #) indicates $p<0.05$; two symbols indicate $p<0.005$; three symbols indicate $p<0.001$.

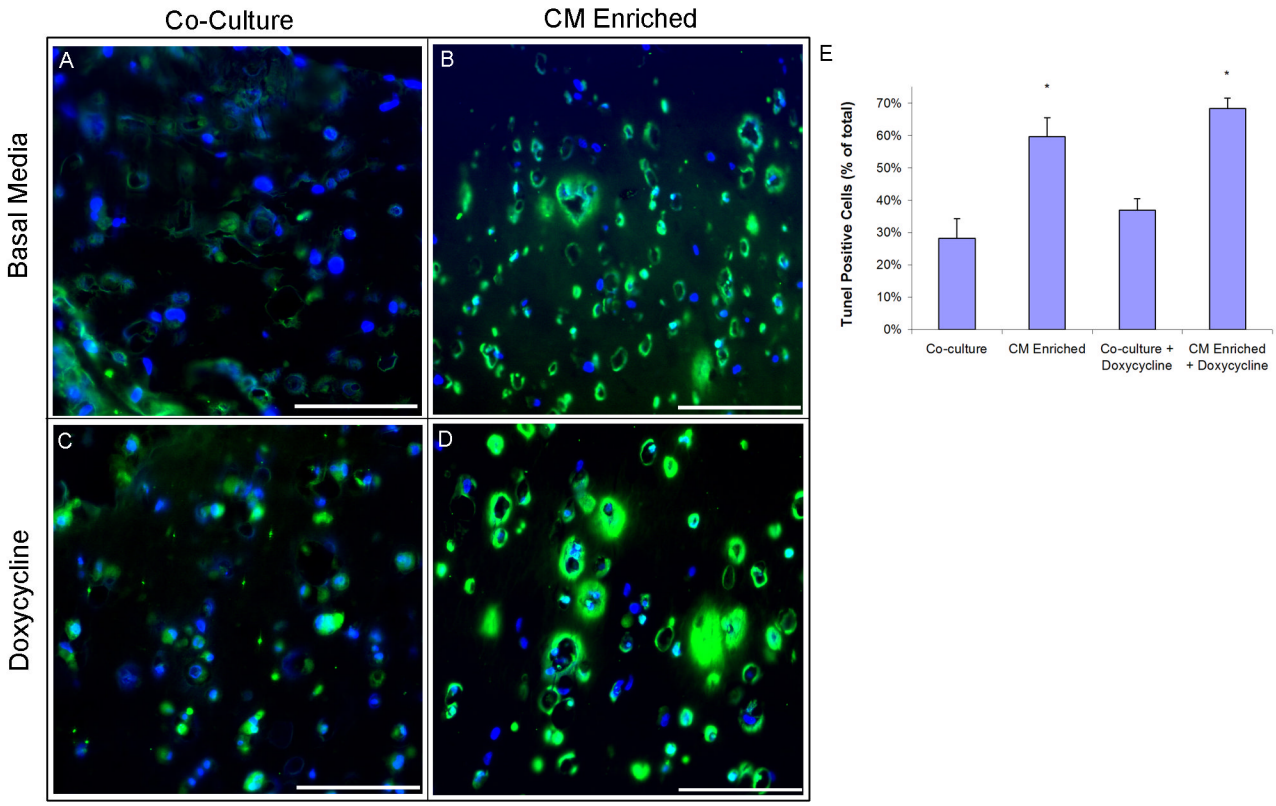

Figure 4.

Immunohistochemical staining for apoptosis in 8-day constructs. Representative images of Co-cultured (A,C) and CM-enriched (B,D) constructs cultured in basal media (A,B) and in the presence of an MMP inhibitor (C,D). Apoptotic (TUNEL-positive) and total (DAPI-positive) cells are colored green and blue, respectively. Scale bars: 100 μm. Data are the mean ± SEM of triplicate samples. Significant difference due to cell population is signified by '*', while significant difference due to MMP inhibition is signified by '#', with $p<0.05$.