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Hybrid Gel Composed of Native Heart Matrix and Collagen Induces Cardiac Differentiation of Human Embryonic Stem Cells without Supplemental Growth Factors

Yi Duan, **Zen Liu**, **John O'Neill**, **Leo Q. Wan**, **Donald O. Freytes**, and **Gordana Vunjak-Novakovic**
Department of Biomedical Engineering, Columbia University, 622 west 168th Street, VC12-234, New York, NY 10032, USA

Abstract

Our goal was to assess the ability of native heart extracellular matrix (ECM) to direct cardiac differentiation of human embryonic stem cells (hESCs) in vitro. In order to probe the effects of cardiac matrix on hESC differentiation, a series of hydrogels was prepared from decellularized ECM from porcine hearts by mixing ECM and collagen type I at varying ratios. Maturation of cardiac function in embryoid bodies formed from hESCs was documented in terms of spontaneous contractile behavior and the mRNA and protein expression of cardiac markers. Hydrogel with high ECM content (75% ECM, 25% collagen, no supplemental soluble factors) increased the fraction of cells expressing cardiac marker troponin T, when compared with either hydrogel with low ECM content (25% ECM, 75% collagen, no supplemental soluble factors) or collagen hydrogel (100% collagen, with supplemental soluble factors). Furthermore, cardiac maturation was promoted in high-ECM content hydrogels, as evidenced by the striation patterns of cardiac troponin I and by upregulation of Cx43 gene. Consistently, high-ECM content hydrogels improved the contractile function of cardiac cells, as evidenced by increased numbers of contracting cells and increased contraction amplitudes. The ability of native ECM hydrogel to induce cardiac differentiation of hESCs without the addition of soluble factors makes it an attractive biomaterial system for basic studies of cardiac development and potentially for the delivery of therapeutic cells into the heart.

Keywords

Tissue engineering; Cardiac differentiation; Human embryonic stem cells; Biomaterial; Native cardiac matrix

Introduction

In the developed world, cardiovascular disease is responsible for the loss of more human lives than all cancers combined. One of the biggest problems is the inability of cardiac muscle to regenerate after massive loss of cardiomyocytes that follows myocardial infarction and often leads to heart failure. Human embryonic stem cells (hESCs) have the ability to differentiate into virtually any cell type including cardiomyocytes and vascular cells. The potential of hESCs to yield all populations of cardiac cells has led to an explosion of studies aimed at developing cell-delivery modalities for heart repair.

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gv2131@columbia.edu .

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hESC-derived repair cells can potentially be implanted into the damaged area of the heart to promote neovascularization and myogenesis. However, the lack of a suitable extracellular environment for cellular adhesion and survival following transplantation has limited cell retention, survival, and integration into the host tissue within the damaged infarct region. To this end, biomaterials derived from decellularized native tissues have been explored as cellular and acellular cardiac patches and injectable hydrogels [1, 2]. It is now of great interest to determine how these biomaterials can be employed to efficiently drive the differentiation of hESCs into mature cardiomyocytes, while supporting cell retention and survival.

Established methods for cardiac differentiation of hESCs utilize soluble factors. For example, Yang et al. [3] developed a staged induction protocol for cardiac differentiation that uses a combination of growth factors at three different stages, to mimic the native development of cardiovascular lineages. This approach yields 50–60% of cardiomyocytes at the end of the induction. Three-dimensional microenvironments in which the cells reside (stem cell niches) are also capable of guiding and directing stem cell differentiation [4, 5]. Development of such microenvironments is important for studying the responses of stem cells to their native extracellular matrix (ECM)— including its composition, stiffness, and topological features and for defining the role of ECM in cell differentiation [6]. With better understanding of the regulatory role of ECM, we may be able to design ECM-based scaffolds that could guide hESC differentiation into cardiomyocytes by mobilizing mechanisms involved in native cardiac development without the use of culture medium supplements.

Hydrogels are one of the most extensively studied types of biomaterials because of their resemblance to native ECM. Hydrogels can be designed to retain the cells, improve cell survival, and enhance cell differentiation and integration with host tissues [7–10]. Components of the native ECMs—collagen, gelatin, hyaluronic acid, and extracts of native ECM have been used in hydrogel form for cell culture [2, 11–13]. For highly contractile skeletal myotubes, an overlay of collagen I hydrogel enhanced myotube to myofiber differentiation in vitro and the production of contractile protein [14]. Injecting collagen I hydrogel with neonatal myocytes into infarcted tissue was shown to improve cardiac ejection fraction, presumably due to better retention and survival of the injected cells [15]. In particular, scaffolds derived from naturally occurring ECMs have the obvious advantage of providing a well-balanced composition of native protein components. In vivo studies have shown regional compatibility and tissue remodeling even in cases when the ECM was derived from non-cardiac tissues such as those of the urinary bladder [16].

Based on the combined results of studies utilizing various types of scaffolds derived from native tissues, we hypothesized that using cardiac ECM will improve the differentiation of hESCs into cardiomyocytes. We addressed two specific questions: (1) whether native cardiac ECM can drive differentiation of human ESCs into cardiac lineages and (2) whether the use of cardiac ECM hydrogel can alleviate the need for supplemental growth factors. To evaluate the responses of embryonic cells to different hydrogels, we cultured embryoid bodies (EBs) and evaluated their morphological changes and the amounts and distributions of cardiac cell lineages. We further examined the effect of the ratio of cardiac ECM and collagen in hydrogel on cardiomyogenic differentiation of hESCs, using gene expression and functional assays.

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Materials and Methods

Human ESC Culture

The human ESCs (NIH code ES02) were obtained from ES Cell International [3]. Briefly, hESCs were grown on a layer of mitomycin-treated mouse embryonic fibroblasts (Invitrogen) in hESC culture medium containing DMEM/F12 (Mediatech, Herndon, VA) and supplemented with 20% knockout serum replacement, 10 mM nonessential amino acid, 200 mM glutamine, 1% penicillin/streptomycin, 0.2 μM β-mercaptoethanol (Sigma, St. Louis, MO), MEF-conditioned medium, and 20 ng/ml bFGF (Invitrogen). Cells were incubated at 37°C and 5% CO_2. Medium was changed daily, and ESCs were split every 5 days using standard procedures [3].

Cardiac Differentiation of Human ESCs

The cardiac differentiation protocol used in our study is based on previously established protocols [3, 17], as shown in Fig. 1. Briefly, human ESCs were feeder-depleted by culture on a thin layer of Matrigel (BD Biosciences, Bedford, MA) in hESC culture medium for 24–48 h. EBs were formed by plating small aggregates of human ESCs in 2 ml basic medium (StemPro34, Invitrogen) containing 200 mM glutamine, 0.4 μM monothioglycerol, 5 μg/ml ascorbic acid (Sigma), and 0.5 ng/ml BMP4 (314-BP, R&D Systems). The factors were added in the following sequence: days 1–4: 10ng/ml BMP4, 5ng/ml bFGF (13256-029, Invitrogen), and 3ng/ml activin A (338-AC/CF, R&D Systems); days 4–8: 10ng/ml VEGF (293-VE, R&D Systems) and 150ng/ml DKK1 (1096-DK/CF, R&D Systems); after day 8: 10ng/ml VEGF, and 5ng/ml bFGF. Cultures were maintained in a 5% CO_2/5% O_2/90% N_2 environment for 12 days of differentiation.

Decellularization of Heart Tissue

Porcine hearts were procured from Yorkshire pigs (65–70 kg) immediately following euthanasia, excess tissue trimmed, blood and debris removed with water, and stored at −80°C for at least 24 h. The hearts were thawed, and then myocardium (left and right ventricles) was sliced into thin (<1 mm) sections (Fig. 2) and decellularized using a modification of our previously established method [18]. Briefly, the slices were initially washed with 2× phosphate-buffered saline (PBS) for 15 min followed by 2 h of 0.02% trypsin, 2 h of 3% Triton X, and 2 h of 4% deoxycholate treatment. After each step, heart slices were washed with 2× PBS for 15 min. Finally, the slices were treated for 1 h with 0.1% peracetic acid, subjected to alternating 1× PBS and dH_2O washes, cut into smaller pieces, and snap-frozen in liquid nitrogen. Frozen pieces were pulverized using a mortar and pestle and lyophilized.

Preparation of Native ECM Hydrogels

The lyophilized powder was digested using a method established in previous studies [19]. Briefly, 1 g of lyophilized ECM powder was mixed with 0.1 g of pepsin in 0.01 N HCl. The solution was allowed to digest for 48 h at room temperature under constant stirring. The final solution was aliquoted and stored at −80°C until use. Gels were prepared by mixing collagen and cardiac ECM with 10× PBS and 0.1 N NaOH at 4°C to yield a final concentration of 4 mg/ml with the appropriate cardiac ECM to collagen ratio. PBS, 10×, and 0.1 N NaOH were used to bring the collagen and cardiac ECM digest stock solutions to neutral salt concentration and pH. The gelation kinetics were determined by transferring 50 μl of the appropriate gel mixture into a 96-well plate at 4°C and transferring the plate into the spectrophotometer which was warmed up to 37°C, and the optical density was measured at 405 nm every 2 min for 60 min.

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Encapsulation of hESCs in Hydrogel

Six experimental groups were formed, by culturing EBs in three different hydrogels (collagen; 25% ECM in collagen; 75% ECM in collagen), with and without supplemental growth factors. At day 4 of differentiation, EBs formed from hESCs were removed from culture, washed with IMDM medium supplemented with antibiotics, and resuspended (0.5 million cells/ml) in either plain culture medium or medium supplemented with growth factors (VEGF and DKK1 that are normally supplemented at days 4–8 stage of culture). Fifty microliters of EB suspension was added to 1 ml of pure collagen (2 mg/ml) solution, or to 1 ml of 75% ECM/25% collagen and 25% ECM/75% collagen hydrogel solutions. The collagen and ECM solutions were sterilized prior to encapsulation under UV light in a laminar flow hood for 30 min at 4°C. Twenty-four-well plates were coated with 50 μl of the indicated hydrogel solution and incubated at 37°C for 30 min to allow for gelation before encapsulating the EBs. After gentle mixing, 250 μl of each EB/gel mixture was transferred (in quadruplicate) to coated wells and likewise incubated at 37°C for 30 min. Fresh medium was then added to each sample and changed at day 8 and day 12 of culture in accordance with the differentiation protocol. Hypoxic conditions were maintained throughout the first 12 days of culture.

Mechanical Properties of Hydrogels

Mechanical properties of hydrogels were characterized as in our previous studies [20]. Briefly, the hydrogel solutions were polymerized in custom-made molds to obtain disks (12 mm in diameter, 3 mm thick) that were transferred between two flat porous platens into a shear-strain controlled rheometer (ARES-LS1, TA instrument, New Castle, DE). A dynamic shear test was performed on a logarithmic frequency sweep (0.1–5 Hz) with shear strain amplitude of 0.1 rad. The complex shear modulus G was calculated from:

$$G=\frac{Td}{2I_p\gamma} \tag{1}$$

where T is the torque response, d is the sample diameter, γ is the sinusoidal shear strain, and I_p is the polar moment of inertia of the cylinder ($I_p = \pi d^4/32$). In general, $G = G' + iG''$, where G' is the storage modulus and G'' is the loss modulus. The complex shear modulus ($|G|$) is therefore calculated as: $|G|=\sqrt{(G')^2+(G'')^2}$, and the phase shift angle (δ) between the applied strain and the torque response is calculated from $\tan(\delta) = G''/G'$ which is called loss tangent.

Immunohistochemistry

For immunofluorescence analysis, hydrogel-embedded EBs were incubated with 0.2% collagenase type I for 1 h at 37°C and dissociated by passing through a 5-ml syringe with a 20-gauge needle for three to six times. Dissociated cells were cultured on chamber slides coated with type I collagen for 2 days, fixed with 4% paraformaldehyde for 5 min, and followed by permeabilization, blocking, and overnight incubation with primary antibodies as previously described [21, 22]. Secondary antibodies Alexa 488 conjugated goat anti-mouse IgG and Alexa 536 goat anti-rabbit IgG (Invitrogen) were then added and incubated for 2 h. Cell nuclei were labeled by Topro3 (Sigma). Fluorescence images were acquired using a confocal microscope (Zeiss, LSM510 Meta). Volocity 5.5 software (Improvision) was used to capture 1 μm Z-stacks to generate extended focus images.

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Gene Expression Analysis

RNA was extracted from culture using TRIzol reagent (Sigma) and standard isolation techniques. Concentration and purity were assessed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, NH). Following conversion to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA), quantitative reverse transcription-polymerase chain reaction was conducted to analyze gene expression according to manufacturer's recommendations. Briefly, TaqMan Gene Expression Assays (Applied Biosystems) were performed in 20-μL reaction volumes with TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and the sample cDNA. Gene expression of TNNT2 relative to GAPDH, the endogenous control, was quantified via a 7500 Fast Real-Time PCR sequence detection system (Applied Biosystems) using the comparative C_T methods [23]. Data were expressed as a fold-change of the ratio in expression of TNNT2 and GAPDH.

Analysis of Contractile Function

EBs grown in hydrogels were observed under phase contrast microscope (Olympus IX81) at 37°C, and 200 frames of videos were taken at frame rate of 35.89 ms/frame. EB size was determined by pixel counting using ImageJ software (NIH) and analyzed with Microsoft Excel. The beating areas were defined as areas with the contraction displacement of >1%. Contraction amplitude was determined as the fractional change of the EB area.

Statistical Analysis

All values are expressed as mean±SD. PCR data were analyzed using one-way and two-way ANOVA. Within each group, growth factor treatment was compared with control (no growth factor) culture using a two-way ANOVA with a Bonferroni post hoc test. To compare each ECM hydrogel group with EBs cultured in collagen hydrogel supplemented with growth factors, a one-way ANOVAwas used using Dunnett's post hoc. A value of $p<0.05$ was considered significant for each test.

Results

Properties of ECM/Collagen Hydrogels

In order to decide which exact compositions of the hydrogel to use, we first characterized the gelation kinetics for four experimental groups: pure ECM, 75% ECM/25% collagen, 25% ECM/75% collagen, and 100% collagen type I hydrogel. The gelation time was nearly three times longer for hydrogels with higher ECM contents (75% ECM) than for 25% ECM and pure collagen (30 min vs 10 min vs 10 min, respectively; Fig. 2a). For pure cardiac ECM without the addition of collagen, the gelation kinetics was too slow (60 min for complete gelling) to allow spatially uniform encapsulation of the EBs. Thus, we focused on hydrogels containing 75%, 25%, and 0% ECM.

The measurement of shear properties of ECM hydrogels (Table 1) showed that the storage shear modulus increased in a linear fashion with the logarithm of loading frequency (Fig. 3b–e). The storage modulus increased when the frequency increases 0.1 to 5 Hz by ~16% for 25% ECM and by ~38% for 75% ECM (Fig. 3b). The loss modulus of 25% ECM hydrogel first decreased with an increase in loading frequency from 0.1 to 0.5 Hz and then continued to increase till 5 Hz, resulting in a bell-shaped curve (Fig. 3c). Both the storage and loss shear moduli changed significantly with the ECM concentration. When the ECM content decreases from 75–25%, both storage and loss shear moduli increased by over six times (Fig. **3**b–c), while the phase shift angle δ or loss tangent remained unchanged (~17° and ~0.3°, respectively, for 75% and 25% hydrogels, Fig. 3d). At 1 Hz, a rate that is close to physiological heart beating rate, the dynamic shear modulus was 61.5±5.9 Pa for 75% ECM

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gel and 8.6±1.5 Pa for 25% ECM gel (Fig. 3b). For comparison, the dynamic shear modulus of 2 mg/ml pure collagen hydrogel is ~25 Pa [24].

Morphology and Cellularity of Hydrogel-Encapsulated EBs

Analysis of EB morphology and cellularity was performed at day 12 after the initiation of cell differentiation. Figure 4 shows representative images for EBs in hydrogels for all six experimental groups (three hydrogels, with and without supplementation of growth factors). Occasional appearance of voids within EBs was attributed to the formation of cysts. In both 75% ECM groups, EBs appeared heterogenous in size (Fig. 4a, b, stars) and tended to group together (Fig. 4a, b). In contrast, EBs in collagen retained their original spherical shape. Representative images of cardiomyocytes in collagen gels with and without growth factors are shown in Fig. 4e, f, respectively.

Cell outgrowth from EBs was observed only in the pure collagen group with supplemental growth factors (Fig. 4f, stars). Supplementation of growth factors did not lead to significant changes in dsDNA content in any of the hydrogel groups. EBs in 75% ECM hydrogel contained twice as many cells as EBs in 25% ECM hydrogels (data not shown). In addition, the EBs in 75% ECM group with supplemental growth factors had larger projected areas than EBs in all other groups (Fig. 4g), in contrast to EBs in hydrogels without supplemental growth factors that better maintained their original spherical shape.

Cardiac Differentiation of hESCs in Hydrogel-Encapsulated EBs

To evaluate cardiac differentiation of hESCs in three-dimensional hydrogels with and without supplemental growth factors, we assessed the mRNA expression of cardiac troponin T (cTnT), a widely used mature cardiac marker, at day 8 and day 12 of differentiation. At day 8, the expression level of cTnT was significantly higher in both the 25% and 75% ECM hydrogel groups without the supplemental growth factors. In contrast, the supplementation of growth factors to the collagen group increased expression of cTnT. Interestingly, the expression level of cTnT in the 75% ECM group without supplemental factors was comparable to that in collagen group with supplemental growth factors (Fig. 5a). By day 12, the effects of supplemental growth factors in 75% ECM group remained significant, in contrast to 25% ECM and collagen groups (Fig. 5b). Also, by day 12 of culture, the expression of cTnT became comparable for all hydrogel groups with supplemental growth factors (Fig. 5b).

Contractile Function of Differentiating Cells

Contractile behavior of cells, a functional indication of cardiac differentiation, was observed in all experimental groups, with and without supplemental growth factors. At day 12 of differentiation, the percent of beating area in both ECM groups was higher without than with the addition of supplemental factors (61.8±6.7% vs. 30.8±3.2% for the 75% ECM gel; 34.8±9.7% vs 13.2±7.1% for the 25% ECM gel) and highest for the 75% ECM group among all hydrogel groups (Fig. 6a). Similarly, the contraction amplitude of cells within EBs cultured in both ECM groups was significantly higher without than with supplementation of growth factors; such effect was not observed for collagen hydrogel group (Fig. 6b). EBs in 75% ECM hydrogels showed markedly higher contraction amplitudes compared with either 25% ECM or collagen hydrogel (3.09±0.17% vs. 1.94±0.55 vs. 1.58±0.13%). Taken together, the measurements of contractile behavior showed that the 75% ECM hydrogel group without supplemental growth factors outperformed all other groups (Fig. 6a, b).

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Expression of Cardiac Markers

To further characterize the contractile cell population, cardiac-specific immunofluorescence staining using distinct marker cardiac troponin I (cTnI) and C×43 was performed. Figure 7 shows that the cells in hydrogels express positive staining for cTnI. Interestingly, only cells in hydrogel without growth factors demonstrated the typical striation patterns indicative of mature sarcomeres (Fig. 7a, c). Strikingly, cells in 75% ECM revealed the most organized distribution of C×43, localized both at the cell periphery and at the perinuclear sites, as compared with scattered localization of C×43 in cells cultured in 25% ECM (Fig. 7a). The expression of C×43 was also upregulated when cells were cultured without the supplementation of growth factors (Fig. 7a, c). In comparison, in both 75% and 25% ECM hydrogels with supplemental growth factors, C×43 demonstrated limited expression among the cTnI-positive cells (Fig. 7b, d), with clusters of other types of cells expressing most of C×43 (Fig. 7d). Notably, the striation structure occupied only 20% of the entire cTnI positive area in 75% ECM hydrogels supplemented with growth factors (Fig. 7b). These results indicate that native ECM promotes the differentiation of hESCs into cardiac lineage and that further supplementation of growth factors did not enhance the efficiency of the differentiation.

Discussion

Our study shows that a cardiac ECM hydrogel, without supplemental growth factors, enhanced the differentiation and maturation of early cardiac progenitors to the extents markedly above those achieved using collagen hydrogels with supplementation of cardiac growth factors. Notably, cardiac ECM showed improved cardiac differentiation at all levels: mRNA, presence and distribution of cardiac markers, and contractile function of the cells. The finding that a hydrogel composed of collagen and native cardiac ECM can replace soluble factors supplemented to culture medium opens a possibility of regulating cardiac differentiation through the design of a hydrogel scaffold composed of native ECM.

Cardiac tissue engineering strategies explored thus far have employed a diverse range of natural and synthetic materials, in combinations with various cell types, from skeletal myoblasts and cardiac fibroblasts to stem cells and resident progenitor cells [25]. What all these materials attempt to provide is a three-dimensional scaffold that mimics the native ECM, to guide and promote myocardial regeneration. By providing the necessary microenvironment, ECM regulates many cellular activities, including migration and differentiation [26–28]. One way to provide the necessary biochemical cues for cardiac regeneration is to use hydrogels derived from native cardiac tissue matrix, mixed with collagen, an approach explored in our study. Collagen type I was chosen as the most abundant ECM protein in the adult heart and a protein shown to promote the growth and survival of cardiomyocytes [29, 30].

The native cardiac ECM is a complex and interdependent network of fibrous proteins, proteoglycans, and numerous supporting molecules [26, 27, 31, 32] that plays a significant role in native development [32, 33]. ECM-based scaffolds have been used to repair partial and full thickness myocardial defects, with promising results in terms of matrix and cellular deposition [34]. Clearly, the hydrogel form of the cardiac ECM, produced from decellularized cardiac tissue [2, 19], will also contain numerous peptides, cytokines and growth factors, digested collagen, and many other components. One limitation of our study is that the composition of the ECM hydrogels has not been characterized. Due to the importance of molecular composition and organization of these hydrogels for regulating cell differentiation, we plan to study their composition and architecture in more detail in our future studies.

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Hydrogels composed of 75% ECM obviously contain large amounts of ECM proteins that might play a significant role in directing the cardiac differentiation of hESCs. It has been reported that laminin, one of the native cardiac ECM proteins, stimulates cardiac differentiation through cell adhesion motifs, whereas fibronectin induces cell growth, migration, cytoskeletal organization and most importantly promotes endothelial differentiation [35]. In laminin A-deficient embryos, the heart is “broken” as a result of dissociation of the pericardial cells [36].

Compositional differences in hydrogels used in the current study (75%, 25%, or 0% ECM, balance collagen) were associated with differences in the mechanical hydrogel properties. The measured decrease in the storage shear modulus with an increase in the fraction of ECM in the hydrogel, in conjunction with no effect on the phase contrast angle (Fig. 3b) implies that the ECM hydrogel contains noncrosslinked proteins of relatively small molecular weights. A soft hydrogel (75% ECM, dynamic shear modulus of ~8.6 Pa) readily supported the early stages of hESC differentiation into cardiomyocytes: mesoderm induction, cardiac mesoderm specification, and cardiovascular lineage expansion. This finding is consistent with previous observations that soft substrates respond synchronically to EB contraction and relaxation, thus allowing effective mechanical and biochemical stimulus transfer between the EB hydrogel [37].

As seen from the morphology of EBs in 75% ECM, there seemed to be a grouping effect that allowed the EBs to interact with each other and beat synchronously (Fig. 4a, b). A similar grouping effect was observed for human mesenchymal stem cells (hMSCs) [38]. When seeded on collagen-coated polyacrylamide gels mimicking the elasticity of the respective natural microenvironment, hMSCs were selectively directed toward myogenic, neurogenic, and osteogenic programs, however, with limited expression of terminal differentiation [39]. In previous studies [40], cardiospheres in hydrogel having a modulus of 31–35 kPa showed high expression of cardiac markers (cTnT and MYH6), whereas the dynamic modulus ranging from 1–8 kPa was needed for chicken embryonic cells to differentiate and mature from mesoderm to adult cardiac lineage on a hyaluronic acid/PEG hydrogel [41]. In our study, a dynamic shear modulus of ~8.6 Pa was optimal for cardiac differentiation of hESCs. The discrepancy of the optimal modulus for cardiac differentiation is likely due to different characteristics of the cells, hydrogel composition, and the differentiation protocol used.

Predictable differentiation and maturation of cardioprogenitor cells into bona fide cardiomyocytes is critical for the development of cell-based therapies for successful cardiac repair. Using EB-mediated differentiation approaches, Keller's group developed a stage induction protocol to differentiate hESCs into cardiac lineages with a yield of 50–60% of cardiomyocytes after 16 days of differentiation [3]. We sought to investigate whether the encapsulation of EBs in a hydrogel derived from native cardiac ECM can be an alternative to the supplementation of growth factors to culture medium. To this end, we encapsulated early EBs (after 4 days of differentiation) in hydrogels with different collagen/ECM compositions, with and without the supplementation of soluble growth factors that are normally used to induce cardiac differentiation. Functional analysis revealed that EBs demonstrated contracting behavior when cultured in all hydrogels groups (Fig. 6). EBs encapsulated in 75% ECM hydrogel, without any supplemental factors, provided the best contractility in terms of percentage of beating area and contraction amplitude among all experimental groups. This result suggests that molecular and possibly mechanical cues provided by the hydrogel matrix could themselves guide cardiac development.

To evaluate whether hydrogels promote the maturation of hESC-derived cardiomyocytes, we investigated the expression of mature cardiac specific transcription factors such as cTnT,

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C×43, and cTnI. Real-time PCR assay of cTnT and immunohistochemical staining of cTnI and C×43 were used to investigate hESC differentiation in hydrogels (Fig. 7). The results clearly demonstrated that cTnT was greatly upregulated at day 8 and day 12 in ECM-containing hydrogels without supplemental growth factors, as compared with hydrogels supplemented with growth factors. Notably, EBs in 75% ECM gel expressed a similar level of cTnT as the ones in collagen gel in the presence of growth factors (Fig. 5). The lack of quantitative analyses of cardiac troponin-positive cells isolated from EBs cultured in different hydrogels, with and without the supplementation of growth factors, is a limitation of the present study and an analysis we plan to conduct in near future.

Only cells in hydrogel without growth factors had become striated myocytes with mature sarcomere (Fig. 7a, c), in comparison to limited striation patterns in hydrogel groups with growth factors (Fig. 7b, d). Subsequently, the lateral localized C×43 was most obvious when cells were cultured in high content ECM without the supplement of growth factors (Fig. 7a). However, there were clusters of other types of cells, most likely vascular [3], expressing most of the C×43. Interestingly, growth factors did not enhance the efficiency of cell differentiation in ECM hydrogels, presumably due to competing effects and nonselective binding of soluble factors to the hydrogel matrix. The current study utilized cTnT as the main molecular marker of cardiac differentiation, in conjunction with functional data. Further characterization of the differentiation/maturation of hESC-derived cardiomyocytes is needed by examining multiple cardiac markers (such as Brachyury and Nkx2.5), analysis of the cell population, and quantification of the striation patterns and cell ultrastructure.

One interesting observation was the inhibition of the differentiation/maturation of cells encapsulated in 75% ECM gel when cultured in the presence of growth factors. We propose two possible explanations for this observation. The native cardiac ECM may contain sufficient bioactive molecules and growth factors to provide signals for EB differentiation. By adding additional growth factors, the differentiation process may have been shifted or altered. Alternatively, supplemented cytokines could attach to native ECM within the hydrogel and have long-lasting effects that are not necessarily beneficial. For example, at induction stage 1, BMP4 helps induce a primitive-streak-like population and mesoderm, while the same factor inhibits cardiac myogenesis at a later stage [42].

In summary, we demonstrate that the native cardiac ECM can actively regulate the differentiation and maturation of early (4-day) cardioprogenitors derived from hESCs. The cardiac ECM alone, without supplemental growth factors, was sufficient to direct cardiac differentiation of hESCs. In particular, a hydrogel containing 75% cardiac ECM and 25% collagen over-performed collagen hydrogels with supplementation of cardiac growth factors with respect to the expression of cardiac genes, presence and organization of cardiac proteins, and contractile behavior of cardiac cells. These results motivate further studies of the mechanisms by which the native ECM hydrogel regulates cardiac differentiation of encapsulated cells and the utilization of native ECM hydrogels as cell delivery vehicle for heart repair.

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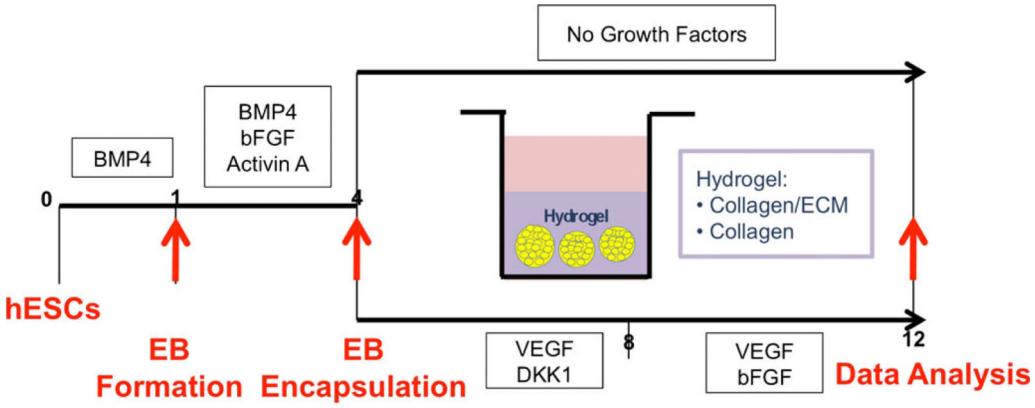

Fig. 1.

Model system for cultivation of human embryonic derived cardiomyocytes encapsulated in hydrogels. The cardiac differentiation protocol of was adapted from Yang et al. [3]. Briefly, human embryonic stem cells (hESCs) undergo the formation of a primitive-streak-like population from day 1 to day 4, the induction and specification of cardiac mesoderm from day 4 to day 8, and the expansion of the cardiovascular lineage from day 8 on. On day 4, embryonic bodies (EBs) were encapsulated in three types of hydrogels, each cultured with or without supplementation of growth factors. Media were changed every 4 days

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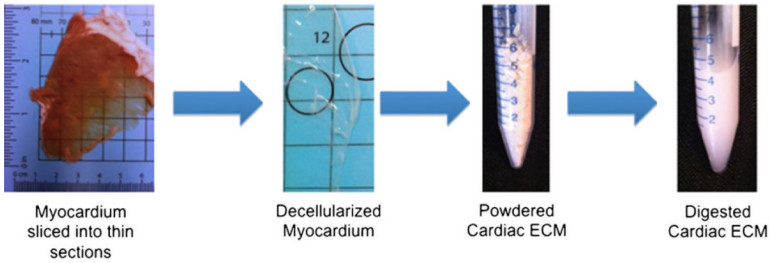

Fig. 2.

Derivation of cardiac ECM digests. Frozen porcine hearts were thawed and sliced into thin sections. The sections were decellularized, crushed, and lyophilized to obtain a cardiac ECM powder. The powder was digested using pepsin to obtain cardiac ECM digests

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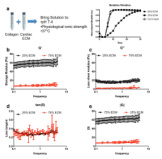

Fig. 3.

Gelation and mechanical properties of cardiac hydrogels. **a** Hydrogel preparation and the measurements of gelation kinetics based on turbidity. Cardiac ECM digest was neutralized at 4°C and brought to 37°C inside a spectrophotometer, and the turbidity was monitored over time. The normalized gelation kinetics is shown as a function of time for pure collagen, 75% ECM, and 25% ECM hydrogels. **b, c** Storage and loss moduli for 75% ECM and 25% ECM hydrogels, respectively. **d** The loss tangent. **e** The dynamic shear modulus as a function of frequency. The dynamic shear modulus of 2 mg/ml pure collagen is 25 Pa [24]. Mechanical data are presented as mean±SD

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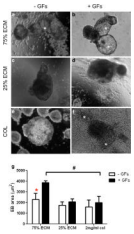

Fig. 4.

Characterization of EBs. **a–f** Morphology of EBs cultured in hydrogels with and without supplemental growth factors. **g** EB sizes under different culture conditions determined using ImageJ in three separate EB differentiation experiments. Data represent mean±SD. *GF* growth factors; *ECM* extracellular matrix; *col* 2 mg/ml collagen gel. #, $p<0.01$

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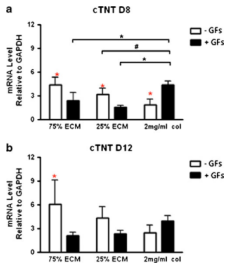

Fig. 5.

Expression of cardiac troponin T (cTnT). Data are shown for three groups of hydrogels with and without supplemental growth factors. Expression in blend gels at day8 (**a**) and day 12 (**b**). GAPDH was used as an endogenous control. Data represent mean±SD. *p<0.001; #p<0.01. *Red star* marks the two-way ANOVA comparison

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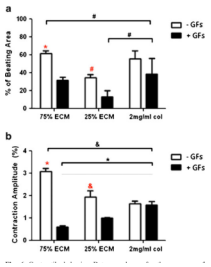

Fig. 6.

Contractile behavior. Data are shown for three groups of hydrogels with and without supplemental growth factors. **a** Beating area. **b** Contraction amplitude. *$p<0.001$; #$p<0.01$; $^{\&}p<0.05$. Red star marks the two-way ANOVA comparison. *GF* growth factors; *ECM* extracellular matrix; *col* collagen gel

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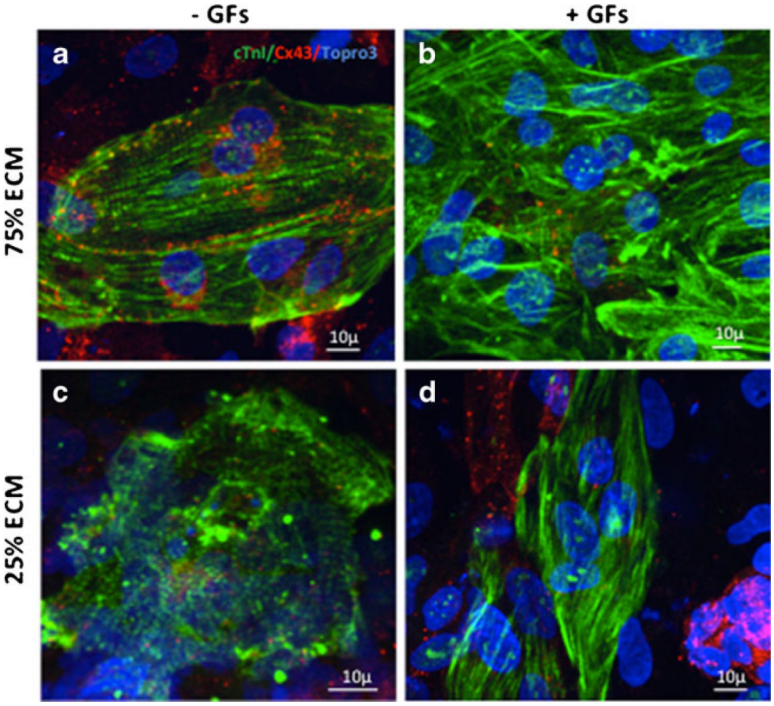

Fig. 7.

Cardiac markers. Confocal images of cardiac marker, troponin I (cTnI, green) and connexin 43 (Cx43, red) staining patterns in human ESC-derived cardiomyocytes cultured in different hydrogels in the absence or presence of growth factors. *Bar, 10* μ

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Table 1

Mechanical properties of the collagen/ECM gel mix

	ECM, mg/ml	Collagen, mg/ml	Shear modulus G',Pa	Loss modulus G'', Pa
75% ECM	3	1	8.17 ± 1.4	2.6 ± 0.5
25% ECM	1	3	59.3 ± 6.6	16.4 ± 1.3

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