

Chemical Modification of Porcine Acellular Extracellular Matrix for Cardiovascular Tissue Regeneration

Yao Wang^{1,2}, Tomer Bronshtein¹, Freddy Yin Chiang Boey¹, Subbu S. Venkatraman¹ and Marcelle Machluf² +

¹ School of Materials Science and Engineering, Nanyang Technological University, Singapore

² Faculty of Biotechnology and Food Engineering, Technion – Israel Institute of Technology, Israel

Abstract. Vascularization remains a critical requirement for the long term survival of engineered tissue constructs, especially thick ones [1, 2]. Such thick constructs for cardiac tissue engineering has been reported by our group [3] and others [4] based on decellularized porcine cardiac extracellular matrix (pcECM) that has been shown to resemble the native tissue both structurally and chemically. The network of inherent vasculature which was largely retained within our pcECM, can be used as a platform for re-endothelialization [5] and neo-vascularization with cell lineages possessing regenerative potential. Endothelial cells alone, seeded onto the ECM, not only attached and survived but also rearranged into typical confluent monolayer morphology with self-alignment. Sequential co-cultures of endothelial cells (HUVEC) and mesenchymal stem cells (MSC) were shown to support the growth of both lineages on the surface and in the vasculature of reseeded pcECM. After ECM treatment with gelatin or fibronectin, cell proliferation increased significantly for both MSCs and HUVECs. Preliminary results showed that future efforts combining co-culture, treated scaffolds and dynamic culture environment may result in re-endothelialization leading to functional blood vessels in thick engineered tissue for cardiac replacement therapy.

Keywords: Cardiovascular, Tissue regeneration, Vascularization, extracellular matrix

1. Introduction

Myocardial infarction (MI), commonly referred to as a heart attack, is mostly caused by blockage of the major blood supply to the cardiac tissue, leading to ischemia and cell death. As adult cardiomyocytes are terminally differentiated, once the heart tissue is injured, the human heart cannot repair itself and a scar tissue is formed. The excess mechanical load on the damaged tissue, which leads to ventricular remodelling, will eventually cause congestive heart failure [6]. While heart transplantation remains the most efficient treatment for end-stage heart diseases, donor shortage and immune rejection still limit its wide application [7]. Hence, alternative approaches have been suggested for heart regeneration utilizing cardiac patches made from biocompatible materials, seeded with expandable and functional human cells, and grown in supportive environment [6].

Cardiac extracellular matrix (ECM), which largely possesses chemical and mechanical properties similar to those of native cardiac tissue, has been widely suggested as a superb scaffold material for cardiovascular tissue engineering [5, 8]. An effective process to isolate acellular ECM from porcine left ventricle wall, in its full thickness (>10 mm), was reported by us to retain the mechanical properties and the major ECM components as well as support the long-term survival of cardiomyocytes and MSCs [5]. Moreover, the ECM was shown to retain the structure of its inherent vasculature that can be used ex- or in-vivo as conduits to feed the tissue bulk, thus overcoming the diffusion barrier limiting survival of cells more than 100 µm away from the nearest blood vessel [9]. Yet, the re-endothelialisation of these conduits remains a critical problem

+ Corresponding author: Prof. Marcelle Machluf. Tel.: +972-4-8293079; fax: +972-4-8293399.
E-mail address: machlufm@tx.technion.ac.il.

requiring optimized conditions to support the attachment, survival, and proliferation of endothelial cells on the ECM vasculature and potentially lead to vasculogenesis and angiogenesis.

Endothelial cells seeded into the vasculature of the ECM were only shown to survive and reach a stabilized phase over time without proliferation, which is necessary for the formation of functional blood vessels. As such, relying on the potential of co-cultures and different ECM components to influence the endothelial cells, we have tested the effect of MSC-HUVEC co-cultures and several ECM modifications on the growth and rearrangement of HUVECs seeded onto the ECM. For practical reasons and to allow efficient and broad screening of a variety of factors, the cells in this case were seeded onto the surface of tissue that was shown to be quite similar to the ECM vasculature.

2. Materials and Methods

2.1. Cell Culture

Human bone marrow derived MSC and HUVEC were both purchased from Lonza (Basel, Switzerland). MSCs were cultured in Minimum Essential Medium Eagle, alpha modification (α MEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Gibco, Langley, OK), 1% L-Glutamine (Gibco), 5 ng/ml bFGF (Invitrogen, Carlsbad, CA), and 1% Antibiotic-Antimycotic (Gibco). HUVECs were cultured in EGM-2 complete medium (Lonza) supplemented with 1% Antibiotic-Antimycotic (Gibco).

2.2. ECM Preparation

Acellular ECM was isolated from porcine left ventricle wall as published before [5]. ECM patch was cut into cylindrical pieces with bottom area of about 0.33cm^2 . ECM slices were then washed with 70% ethanol, followed by PBS and EGM-2 washes. Gelatin (4 mg/ml), fibronectin (10 $\mu\text{g/ml}$), and laminin (100 $\mu\text{g/ml}$) coating solutions were prepared by dissolving protein powders in PBS. ECM slices were immersed in one of the coating solutions for 24 hours. Un-coated ECM served as control.

2.3. Cell Seeding

Prior to seeding, ECM slices were individually placed into 96-well low binding plate (NUNC, Roskilde, Denmark). For simultaneous co-culture, 150,000 MSCs and 150,000 of HUVECs were seeded together in a mixed suspension on each ECM slice. For sequential co-culture, HUVECs were seeded 7 days after MSCs. For single culture, 300,000 HUVECs or 300,000 MSCs were seeded separately. Re-seeded ECM slices were cultured in 24-well low binding plate (NUNC) in EGM-2 for 21 days. Medium was replaced every second day.

2.4. AlamarBlue™ Assay

Re-seeded ECM slices were incubated in EGM-2 medium with 10% AlamarBlue reagent (Invitrogen) for 5 hours. The fluorescence intensity was measured with Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. ECM with no cells served as control. The viability of the cells was calculated from the AlamarBlue readings which were taken every second day.

2.5. Cell Labeling with DiI

Cells were harvested and incubated with 12.5 μM non-specific membrane labelling dye DiI for 30 minutes at 37°C. To remove excess dye, cells were then plated and cultured overnight prior to seeding on ECM.

2.6. Statistical Analysis

Averaged results are presented as mean \pm SE of $n\geq 3$. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistically significant difference is defined for $p<0.005$.

3. Results

3.1. Cell Morphology

Confocal microscopy imaging showed that HUVECs attached and aligned 7 days after seeding (Fig. 1a) while MSCs were relatively less arranged but more densely packed against each other (Fig. 1b). When co-cultured together (Fig. 2c), DiI-labelled HUVECs were seen even more uniformly arranged, better spread and aligned than singly cultured ones.

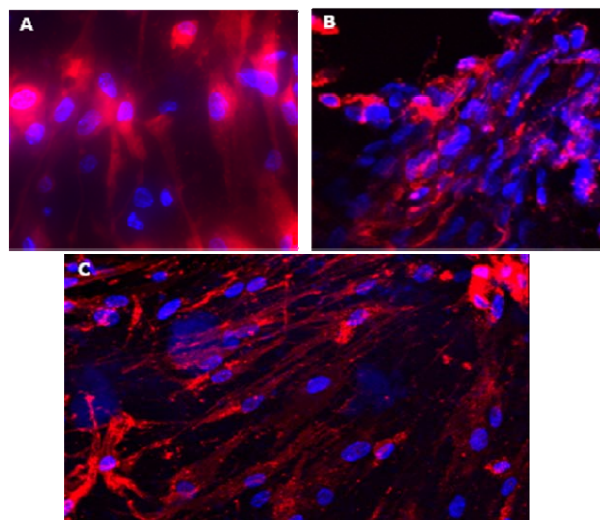
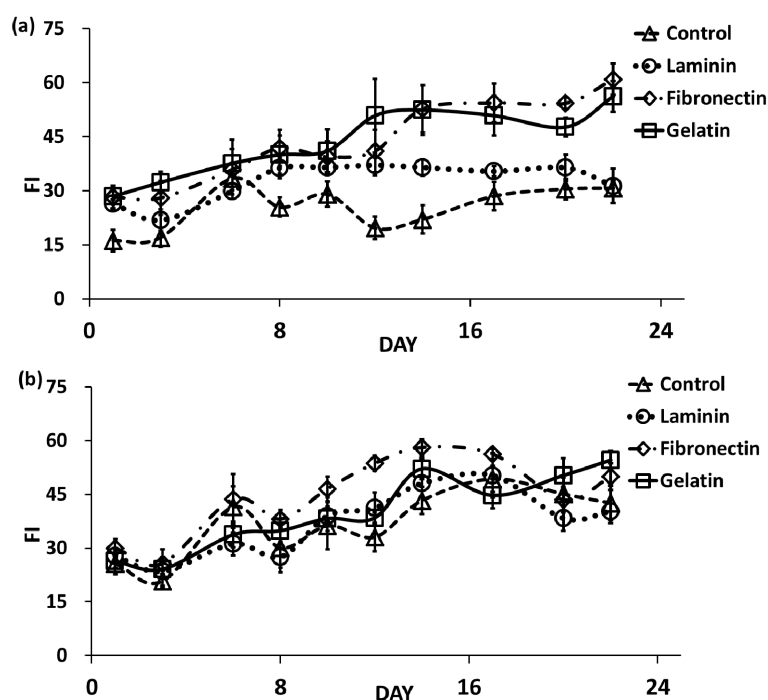


Fig. 1: **Rearrangement of HUVEC and MSC reseeded on ECM.** Confocal images of HUVEC (a), MSC (b), and simultaneous co-culture at 1:1 ratio (c) cultured for 7 days. Cells were pre-labelled with DiI (red) prior to seeding and counterstained with DAPI (blue) for cell nuclei. In the co-culture, only the HUVECs were pre-labelled with DiI.

3.2. Cell Proliferation

The proliferation of HUVEC and MSC reseeded on ECM treated with laminin, fibronectin, and gelatin was measured using AlamarBlue. Significant improvement in HUVEC growth was observed on all treated ECM samples, especially the ones treated with fibronectin and gelatin (Fig. 2a, $p < 0.001$). MSCs alone exhibited better proliferation only on the fibronectin-treated scaffold (Fig. 2b) while the proliferation of MSC-HUVEC co-cultures, similarly to HUVEC alone, was best following fibronectin and gelatin coating (Fig. 2c). Sequential co-culture approach led to improved cell attachment and growth compared to simultaneous co-culture and single culture (unpublished data). However, no additional improvement was observed with ECM treatment (Fig. 2d).



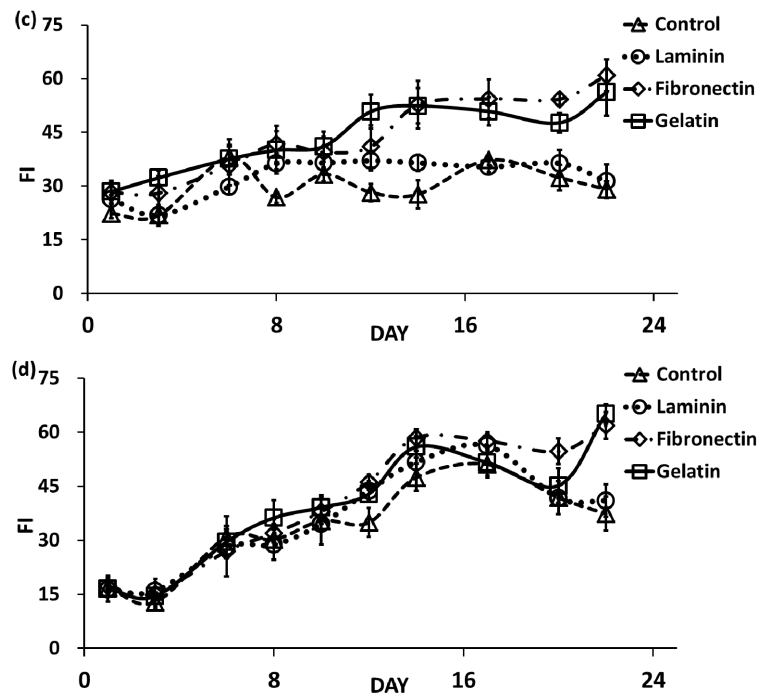


Fig. 2: Cell proliferation over 21 days on biochemically modified ECM scaffold. HUVEC (a), MSC (b), simultaneous co-culture (c), and sequential co-culture (d) on untreated ECM pieces (control) and ECM treated with laminin, fibronectin, and gelatin. Cell viability is presented as AlamarBlue Fluorescent intensity (FI).

4. Discussion

Multipotent MSCs, harbouring therapeutic potentials, were shown to support the attachment of endothelial cells and enhance angiogenesis [10] making them ideal candidates for applications involving cardiovascular tissue regeneration. Our previous studies demonstrated that when being co-cultured on tissue culture plate, MSCs and HUVECs exhibited some mutual effects which influence the overall population dynamics and growth over time. In a different study, we showed that MSCs enhanced the proliferation of HUVECs on 3-dimensional ECM scaffold using sequential co-culture (unpublished data). Here, confocal images showed that MSCs affected the alignment and morphology of HUVECs on ECM, which could result from physical contact or signalling proteins secreted from the cells. Chemical modification using various proteins to coat the ECM revealed that one or two types of proteins are required for the attachment and proliferation of each cell type. Fibronectin coating that led to significantly improved growth of both singly and co-cultured cells may be the best choice among the three.

5. Conclusion

Results from this study validated the advantages of co-culturing MSCs and HUVECs and the potential of acellular ECM to be used as scaffold material for cardiovascular tissue engineering. With biochemical modification, ECM can support the growth and stabilization of endothelial cells over time - the most important requirement for vascularisation and angiogenesis. Effective regeneration of thick tissue with functional blood vessels also requires supportive environment for sufficient oxygen and nutrients supply. Dynamic culturing with bioreactors can provide recellularized tissue with nutrient and waste exchange, physiological environment mimicking their natural condition, and is currently being studied by our group. With the combination of precise cell selection, supporting scaffold, and accommodating culture environment, re-generated thick cardiac tissue with functional blood vessels can serve as an optimal solution for cardiac replacement therapy.

6. References

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