Research Article

Polymer microfiber meshes facilitate cardiac differentiation of c-kit+ human cardiac stem cells

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ABSTRACT

Electrospun microfiber meshes have been shown to support the proliferation and differentiation of many types of stem cells, but the phenotypic fate of c-kit+ human cardiac stem cells (hCSCs) have not been explored. To this end, we utilized thin (~ 5 μm) elastomeric meshes consisting of aligned 1.7 μm diameter poly (ester-urethane urea) microfibers as substrates to examine their effect on hCSC viability, morphology, proliferation, and differentiation relative to cells cultured on tissue culture polystyrene (TCPS). The results showed that cells on microfiber meshes displayed an elongated morphology aligned in the direction of fiber orientation, lower proliferation rates, but increased expressions of genes and proteins majorly associated with cardiomyocyte phenotype. The early (NK2 homeobox 5, Nkx2.5) and late (cardiac troponin I, cTnI) cardiomyocyte genes were significantly increased on meshes ($Nkx=2.5$ 56.2 ± 13.0, $cTnI=2.9 \pm 0.56$,) over TCPS ($Nkx2.5=4.2 \pm 0.9$, $cTnI=1.6 \pm 0.5$, n=9, $p < 0.05$ for both groups) after differentiation. In contrast, expressions of smooth muscle markers, Gata6 and myosin heavy chain (SM-MHC), were decreased on meshes. Immunocytochemical analysis with cardiac antibody exhibited the similar pattern of above cardiac differentiation. We conclude that aligned microfiber meshes are suitable for guiding cardiac differentiation of hCSCs and may facilitate stem cell-based therapies for treatment of cardiac diseases.

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1. Introduction

Cardiovascular diseases are the leading cause of mortality throughout the world. Nearly 85.6 million adults have at least one type of cardiovascular disease, and about half are under the age of 60 [1]. Furthermore, myocardial infarction is the most common cardiovascular disease accounting for more than 40% of cardiovascular disease-related deaths [1]. To date, there are no efficient means to treat these diseases. However, previous animal studies and human clinical trials have demonstrated that stem cell-based therapies hold great potential to repair or regenerate the damaged myocardial tissues, thus alleviating the underlying cause of ischemic heart failure [2,3].

At present, various types of resident cardiac stem cells, such as side-population, c-kit positive, Islet-1 positive, Sca-1 positive, SSEA-1 positive stem cells, as well as cardiosphere-derived stem cells have been identified in animal and human heart tissues [4]. Among these, c-kit+ cardiac stem cells (CSCs) appear to be one of the most promising cells types for improving cardiac function and reducing infarct size in both animal models of myocardial infarction and human studies [5–7]. Further, human c-kit+ CSCs (hCSCs) possess both self-renewal potential and the capacity to differentiate into cardiomyocytes, smooth muscle, and endothelial cells

(ECs) *in vivo* [8]. Recently two clinical trials [6,9] using the intracoronary infusion of autologous hCSCs or cardiosphere-derived cells (which also expresses c-kit marker) reported increased viable myocardium, elevated cardiac function, and reduced infarct size in patients with heart failure after myocardial infarction. Although the efficacies remain a source of debate and the mechanisms for improved cardiac function is poorly understood [7,10], the overall outcomes from these and many other animal studies from different laboratories are still consistent and promising[11]. However, in all stem cell-based *in vivo* studies, the low cell retention, low cell survival, and very limited percentages of *in vivo* cardiomyocyte differentiation following transplantation remains the major limitation in the field [12–14]. To overcome the above issues especially cell death, various interventions have been examined, including pretreatment with heat shock and anti-inflammatory compounds, forced over-expression of anti-apoptotic proteins, addition of free radical scavengers to the culture medium, and the co-delivery of extracellular matrix molecules [15–17]. Combinatorial use of such approaches has been shown to markedly enhance cell survival rate, but low cell retention and engraftment still remain significant problems.

Cardiac tissue engineering has offered new strategies to overcome these problems. Engineered cardiac patches, 3D biomaterial constructs, and bioreactor-conditioned scaffolds are a few examples utilized in the field [18–20]. To enhance cell retention and survival rate, the biomaterial components of these approaches have been designed to mimic the properties of the cardiac extracellular matrix (ECM), such as high surface areas for display of adhesive ligands, and nanoscale architectures with topographical features to affect cell morphology and orientation [18,20,21]. One widely-used approach has been to combine synthetic nanofibers with natural ECM proteins, and examples include composites consisting of aligned poly(glycerol sebacate) (PGS) nanofibers in gelatin hydrogels [22], polycaprolactone (PCL) nanofiber substrates coated with ECM proteins (*e.g.,* collagen types I, IV, fibronectin, or laminin) [23], and decellularized bovine pericardium ECM coated with PCL-chitosan nanofibers [24]. In general, nanofiber scaffolds prepared by electrospinning have demonstrated a superior capacity for guiding cell adhesion, migration, morphology and differentiation when compared to other types of scaffolds [25–32]. Obviously, the biocompatibility and degradability of polyesters, such as PCL, PGS, poly(ester-urethane urea) (PEUUR), makes them well suitable for applications in tissue engineering and regenerative medicine. Among which, PEUUR family shows excellent elasticity, tunable mechanical properties and degradation rates, and degradation products that are not cytotoxic [33–35]. For example, electrospun PEUURs have been shown to support adipose-derived stem cell differentiation into the adipogenic lineage and to improve mesenchymal stem cells adhesion and alignment [35,36].

In the present study, we examined whether aligned electrospun PEUUR microfiber meshes could be potentially used like engineered cardiac patches to maintain hCSCs. Specifically, we examined the impact of PEUUR microfiber meshes on hCSC attachment, proliferation, morphology, and *in vitro* differentiation relative to standard tissue culture polystyrene (TCPS) dishes [6,7]. Gene and protein expression of cardiac linage makers were measured to identify three types of cardiac cells: cardiomyocytes, smooth muscle cells, and ECs.

2. Materials and methods

2.1. PEUUR synthesis, fabrication of microfiber meshes, and assembles of culture

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. A degradable elastomeric segmented PEUUR was synthesized from 2000 Da PCL diol, 1,6-diisocyanatohexane, and 1,3-propanediol bis(4-aminobenzoate) as described previously [36]. For electrospinning, an 11% (w/v) PEUUR solution in hexafluoro-2-propanol was placed in a 3 ml disposable syringe fitted with a 22 gauge blunted stainless steel needle. The collector was a 7.5 cm diameter cylindrical mandrel covered with aluminum foil and onto which polydimethylsiloxane (PDMS) strips (0.2 cm thick × 0.5 cm wide) were arranged axially at 1 cm spacing (circumferentially). Silicone medical adhesive Type A (Dow Corning, Midland, MI) was coated on the PDMS strips prior to electrospinning to adhere deposited fibers. Aligned fibers were collected between PDMS strips by rotating the mandrel at 1500 rpm, applying a DC voltage of +15 kV to the needle, and delivering the PEUUR solution at a rate of 0.8 ml/h using a syringe pump. The electrospinning process was performed at room temperature (RT) and at a humidity of 50%. Meshes were vacuum dried to remove any residual solvent. (Resultant meshes are elastic with tensile moduli of ~9 MPa [36]. Although their rate of degradation has not be determined, they are expected to degrade over the course of several months, similar to the PEUURs synthesized by Kavlock et al. [37].

Next, electrospun meshes −5 mm wide and including the two adjacent PDMS strips – were cut from the foil covered mandrel and the PDMS strips glued to 1.8 cm^2 plastic coverslips such that the meshes were taut and elevated 2 mm above the coverslips (Fig. 1A, a1 and a2). Meshes were then transferred to 12-well culture plates and sterilized by exposure to UV for 4 h before seeding cells.

2.2. Cell culture and cardiac differentiation

hCSCs were isolated and cultured according to our previously published protocols [38]. Briefly, patient heart samples (*e.g,* atrial appendages) were obtained as discarded tissues from local hospitals. A written consent agreement was obtained for collection of discarded atrial appendages by the hospitals and all procedures were approved by the Institutional Review Board (IRB) of Virginia Polytechnic Institute and State University for human subject research. Donor confidentiality was maintained at the hospitals and no patient identification information or medical history was collected according to the approved protocol. Cells were cultured in hCSC medium consisting of consisting of Ham's F12 (ThermoFisher Sci, Grand Island, NY), 10% FBS (JR Scientific, Woodland, CA), 10 ng/ml human bFGF (PeproTech, Rocky Hill, NJ), 0.2 mM L-glutathione, and 0.005 U/ml human erythropoietin (both from Sigma-Aldrich). Cells were then incubated at 37 °C in a 5% CO_2 incubator with medium change every other day. After primary cultures reached ~85% of confluence, cells were lifted with TrypLE express enzyme (ThermoFisher Sci) and 5000 cells within 40 μl hCSC medium as a single droplet were added onto the surface of each microfiber mesh (Fig. 1B, b1). Cell-seeded meshes were maintained in the incubator for 4 h before an additional 1 ml of hCSC medium was added. For comparison, hCSCs were also seeded at 5×10^4 cells on the bottom of 12-well TCPS plates (For both micro-fiber meshes and TCPS, initial cell densities were approximately same as of 1.4×10^4 cells/cm^2). The preliminary data indicated that by using this method [38] we are able to obtain > 90% c-kit$^+$ hCSCs from each patient's heart tissue (data not shown). These high pure c-kit$^+$ hCSCs are used in all following experiments. The detail characterization of isolated c-kit$^+$ hCSCs using the same protocol has been previously published [38].

To induce the cardiac differentiation, the hCSC medium was replaced with cardiac differentiation medium (89% Ham's F12, 10% FBS, 1% antibiotic, and 10 μM 5-azacytidine) 2–3 days after seeding. The cells were cultured in the differentiation medium for 3 days with daily medium changes. Thereafter cells were then