Effect of Electrospun Combinatorial Polymer Composition and Alignment on the Differentiation of Human Induced Pluripotent Stem Cells

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BRIEF. With growing evidence that the extracellular matrix plays a crucial role in stem cell differentiation, induced pluripotent stem cells were cultured on distinct electrospun polymer fibers of varying alignment for four days.

ABSTRACT. Although embryonic stem cells (ESCs) have a variety of merits, disadvantages such as availability and ethical controversy have hampered their use for cell therapy. New breakthroughs in the reprogramming of human somatic tissues to generate induced pluripotent stem cells (iPSCs) have allowed researchers to overcome the complications associated with ESCs, yet have the pluripotency as ESCs. Since there is accumulated evidence that topological and mechanical cues in the extracellular matrix (ECM) play important roles in cell differentiation, we hypothesized that molecular profiles of iPSCs cultured on aligned ECM-mimicking substrates may be different from ones cultured on unaligned substrates. To test this hypothesis, combinatorial polymers composed of three subunits, poly(ε-caprolactone) (PCL), poly(ethylene glycol) (PEG), and carboxylated-PCL (cPCL), were used to alter physicochemical and biological properties of test culture substrates. Aligned and unaligned fibers were created by electrospinning and human iPSCs were reprogrammed. We determined the effects of the polymer composition as well as fiber alignments on the differential and molecular profiles of iPSCs through biochemical, cell biological and engineering methods.

INTRODUCTION.

Stem cell therapy for myocardial infarction, more commonly referred to as heart attacks, is an innovative method for treating patients due to the loss of cardiac tissue, which does not possess the ability to regenerate [1-3]. Embryonic stem cells (ESCs) have been considered a potential cell source for stem cell-based therapies as they are pluripotent, meaning they can differentiate into all cell types of the human body, including cardiomyocytes (cardiac muscle cells) [4-6]. ESCs, however, have had limited use in therapies due to the ethical concerns associated with obtaining the cells as well as their limited availability.

Recently, human induced pluripotent stem cells (iPSCs) have been developed as a new potential cell source [7]. iPSCs are generated by reprogramming terminally differentiated somatic cells back into their embryonic state. These cells are pivotal in the ongoing research for stem cell therapy as they have the same differentiation potential as ESCs but lack the controversies and other limitations associated with ESCs such as availability and source [8]. Especially since iPSCs are derived from a specific patient, they additionally ensure minimal body rejection [9].

The extracellular matrix (ECM) is known to provide structural support for cells but there has been growing evidence that it also provides topological and mechanical cues that may play an important role in stem cell differentiation [10-12]. In order to observe this phenomenon, a previous study examined the effects of the ECM by using combinatorial polymers composed of six ratios of three subunits: poly(ε-caprolactone) (PCL), poly(ethylene glycol) (PEG), and carboxylated-PCL(cPCL), to alter the physicochemical and biological properties of test culture substrates [13]. Results from culturing mice-derived (murine) embryonic stem cells on these polymers showed that the differentiation of the cells and the viability of the derived cardiomyocytes were affected by the ECM-mimicking electrospun fibers and the composition of the polymers [13].

In this prior research, the polymer properties of the electrospun combinatorial polymer solutions were observed, but to date, there has been no investigation into the role of polymer alignment on cell phenotype. As muscle tissues are characterized by alignment, especially cardiac tissue, we investigated the effects of polymer composition and alignment of the fibers on the differentiation of iPSCs [14]. We hypothesized that the alignment of three different combinatorial polymer compositions, 100%PCL, 4%PEG – 96%PCL, and 10%cPCL – 90%PCL, would have varying effects on the differentiation of iPSCs from the unaligned fiber samples. We also hypothesized that iPSCs cultured on fibers would differ from those cultured on polymer films. These effects were investigated using biochemical, cell biological, and engineering methods. Results from this experiment may lead to a better understanding of what factors in the ECM drive the iPSCs towards a certain lineage and would allow researchers to produce differentiated iPSC derived cell lines for stem cell therapies.

MATERIALS AND METHODS.

Electrospinning.

The nanoscale fibers were fabricated using 12.5 wt% solutions using different combinatorial polymers dissolved in 4:1 volume ratio of chloroform to methanol: poly(ε-caprolactone)(PCL) polymer, 4% polyethylene glycol(PEG)-96% PCL polymer, and 90% PCL-10% carboxylated-PCL(cPCL) polymer. Unaligned fibers were electrospun by using a flow rate of 0.7 mL/hr and having the tip of the needle placed 3 cm from a grounded mandrel rotating at 1350 rpm with attached cover glasses. A high voltage of 6.0 kV was applied to the needle tip, resulting in fibers that were collected on the cover glasses on the mandrel.

Aligned fibers were electrospun using a specialized parallel plate method (SUP) as modeled by figure S1 [15]. The parameters used are described in Table 1.

Table 1. Aligned Electrospun Fiber Parameters - The data presented here show an average for each category as external factors such as temperature, humidity, light, and weight percentage all affect the ideal parameters each day. The distance of the needle from the device was kept constant at 3cm.

Polymer Type	Time (aligned)	Voltage (aligned)	Flow rate (aligned)	Time (unaligned)	Voltage (unaligned)	Flow rate (unaligned)
PCL	20 minutes	4.2-4.4 KV	0.07-0.08 mL/hr	30 minutes	6.5 KV	0.8 mL/hr
4% PEG-PCL	35 minutes	3.7-4.0 KV	0.06-0.07 mL/hr	40 minutes	9.5 KV	0.75 mL/hr
10% cPCL	20 minutes	4.4-4.49 KV	0.06 mL/hr	30 minutes	11 KV	1.0 mL/hr

Scaffold Imaging and Analysis.

Scanning Electron Microscopy (SEM) samples were sputter coated with gold for 50 seconds to give conductivity to the non-conductive fibers. Once the loaded samples were in the microscope, image zoom was used to focus the electrons on the sample. Images were taken at various magnifications and voltages.

SEM images were then imported into ImageJ (National Institute of health, Bethesda, MA) for analysis. Average angle of the fibers, thickness of the fibers, and spacing between the fibers were determined. The average and standard deviations were calculated in Excel (Microsoft, Redmond, WA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).

For qRT-PCR, 18ng cDNA was mixed with SYBR Green Master Mix (Bio-Rad) and 500nM mRNA-specific stemness primers (forward and reverse), totaling 20µl in each well of a transparent 96-well plate. Samples were then amplified in a CFX96 qRT-PCR machine (Bio-Rad) with the following steps: 3 minutes at 95°C to denature DNA, followed by 45 steps of 95°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), and 72°C for 30 seconds (extension). Genes of interest included *Sox2, NANOG,* and *PECAM-1*. Expression of stemness genes was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For electrospun scaffold samples, n = 3 biological replicates were used for qRT-PCR whereas n = 2 replicates were used for spin-coated and glass samples. Data were analyzed using Bio-Rad software and results are presented as mean ± standard error of the mean (SEM).

Immunofluorescence Staining.

On day 4, the cells were fixed and permeabilized with 4% paraformaldehyde in PBS and 0.2% Triton X-100 in PBS respectively for 20 minutes at room temperature. The solution was removed and the cells were blocked with 10% goat serum in PBS for 2.5 hours at room temperature. After removing the blocking PBS, 100µL of 0.0016µM light-sensitive Hoeschst dye was added for five minutes. Then 100µL of 0.16µM AlexaFluor488-conjugated Phalloidin (Life Tech, Carlsbad, CA) was added and incubated at room temperature for 20 minutes. After the dyes were removed, the cells were washed once in PBS for 10 minutes and 200µL of PBS was added to the cells for use when imaging.

Statistical Analysis.

In all experiments, results are presented as a mean ± standard error of the mean. Results from each experiment were initially analyzed using single factor analysis of variance and comparisons between individual sample groups were then performed using an unpaired Student's *t*-test. For all statistics, $p < 0.05$ was considered statistically significant.

Supplemental Information.

Please refer to the supplemental information for methods pertaining to spin coating, cell culture preparations, RNA extraction, and cDNA synthesis.

RESULTS.

Electrospun Scaffolds.

The SEM images confirmed uniform, dense coating over the entire cover glass surface (figure 1). Results indicated that for the aligned fibers, there was no significant difference in the range of angles from the alignment direction, fiber diameter (thickness), or fiber spacing between the different polymer groups (figure S2). The results for the aligned samples revealed a high degree of uniform alignment in all regions, as shown in figure 1. In addition, the images reveal that for the aligned samples, the fibers are a single, dense layer rather than multiple layers. This results in a flat topography on the samples.

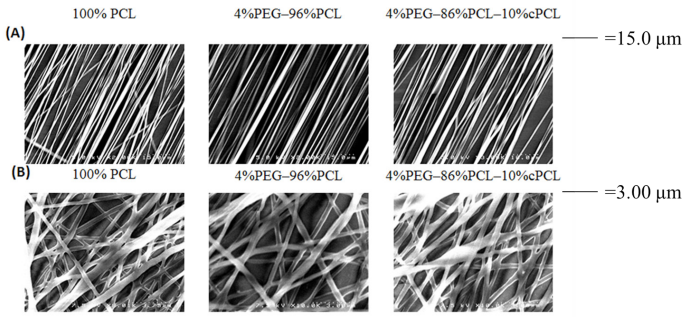

Figure 1. Scanning Electron Microscopy Images of Electrospun Fibers - (A) Aligned samples of the three combinatorial polymers used imaged using the Scanning Electron Microscope (SEM). As can be seen, the fibers are very aligned and form similar angles with the horizontal. (B) Unaligned samples of the three combinatorial polymers imaged with the SEM. The images show that the fibers show no correlation and are completely random.

Immunofluorescence Staining.

On day 4, the cells were fixed and stained with Hoechst, a nuclear stain, and phalloidin, which selectively stains the actin cytoskeleton. From figure 2, clear distinctions can be made from the aligned and unaligned samples. For the aligned samples, especially the 100% PCL, the iPSC cytoskeleton eleongated along the fibers. The cytoskeleton of the unaligned fibers, however, remained dense and compact. The aligning of the cytoskeleton was also observed on the 4%PEG – 96% PCL fibers. The cytoskeleton was visible and alignment was apparent, but compared to the 100% PCL, there were less elongation and alignment. The 90%PCL – 10%cPCL had samples that were very compact and the cytoskeleton was barely visible. The alignment of the fibers did not seem to have effects on the cells. The spin coated samples for every polymer resulted in cytoskeletons that were spread in all directions. Compared to the unaligned samples, the spin coated samples had cytoskeletons that were more fluid (i.e. less defined in terms of directionality).

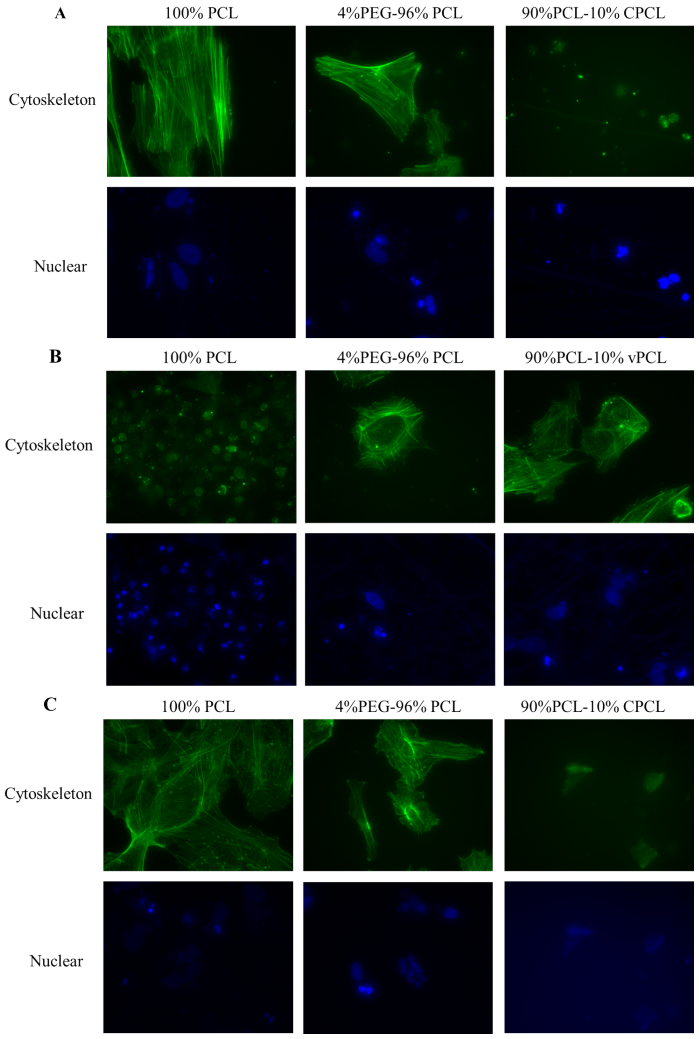

Figure 2. Immunofluorescence Staining – On d-4, a sample from each matrix type was imaged. The cytoskeleton is represented in green and the nucleic material is represented in blue. (A) Aligned samples of each polymer type. It can be observed that 100%PCL and 4%PEG – 96%PCL had cytoskeletons that elongated along the fibers. The 90%PCL – 10%cPCL showed relatively dense cytoskeleton. (B) Unaligned samples for each polymer type. The unaligned fibers had cells that were more compact cytoskeletons that had no uniform direction. (C) Spin coated

samples for each polymer type. The images show cytoskeleton that does not have uniform direction, but elongated. The cytoskeleton in the spin coated sample for cPCL remained dense.

Gene Expression Testing and Statistical Analysis.

The results from the gene expression test involving the three stemness markers, *NANOG*, *Sox2*, and *PECAM-1*, revealed stark and significant differences for the iPSCs cultured upon the various substrates (Figure 3). The three graphs follow similar trends for each scaffold type: spin coated samples for each polymer were generally lower in gene expression as compared to both the glass control and either format of the electrospun fibers. As shown by statistical analysis, although there was a slight up-regulation in aligned samples as compared to unaligned samples for all four stemness markers, no significance was observed ($p > 0.05$). But when the spin coated samples of each polymer were compared to the unaligned samples, significance was observed for the 100%PCL and 4%PEG – 96%PCL ($p < 0.05$). What was unique was that no significance was observed

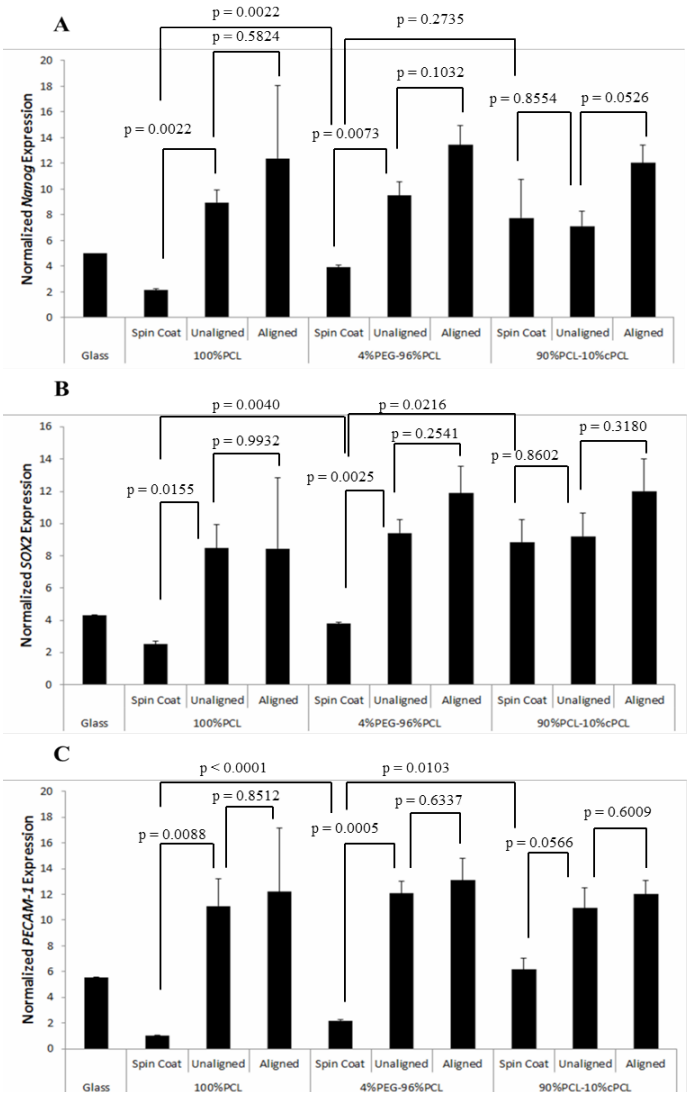

Figure 3. Gene Expression Test - (A) *NANOG* expression was the highest for the aligned samples of each polymer. For 100%PCL and 4%PEG – 96%PCL, the unaligned was second in expression. However, in 90%PCL – 10%cPCL, the spin coated sample expressed more of the *NANOG*. (B) *Sox2* expression had the same trends as the *NANOG*. (C) *PECAM-1* expression is similar to *NANOG* and *Sox2*. However, the spin coated samples decreased.

for the 90%PCL – 10%cPCL when the spin coated samples were compared to the unaligned samples ($p > 0.05$). This indicates that the cPCL component of the combinatorial polymer up-regulates gene expression.

Further analysis revealed that for *Sox2* and *PECAM-1*, significance existed between spin coated samples of 100%PCL and 4%PEG – 96%PCL and between 4%PEG – 96%PCL and 90%PCL – 10%cPCL. For *NANOG*, the 90%PCL – 10%cPCL seemed to have a much higher gene expression in relation to the 4%PEG – 96%PCL, but no significance was observed.

Although *PECAM* is a known endothelial marker, its presence at early embryonic stages has been described [16]. Since a trend following that of *NANOG* and *Sox2* was observed for *PECAM*, the data suggest that this early marker, which is present in the inner cell mass of a developing embryo, might be of critical importance in the iPSCs and is influenced by polymer composition and format.

Supplemental Information.

Please refer to the supplemental information for result pertaining to cell alignment, attachment, and confluency.

DISCUSSION.

The results from the SEM images showed that the three combinatorial polymers were all highly aligned in the aligned samples and random for the unaligned samples. Although the average thickness and spacing varied, the differences were diminutive, meaning the cells differentiated solely based on the alignment and polymer composition. The results from the immunofluorescence staining demonstrated that human induced pluripotent stem cells are responsive to the extracellular environment. The cytoskeleton of the cells aligned and elongated in agreement with the fiber axis of the aligned samples of 100%PCL and 4%PEG – 96%PCL, and slightly in 90%PCL-10%cPCL. iPSCs cultured on the unaligned polymer samples showed cytoskeletons that were non-uniformly dispersed. The cytoskeleton of the unaligned samples elongated in a multitude of directions, unlike the cytoskeleton of the aligned samples. For the spin coated samples, since there is only a sheet of polymer, the cells were not influenced to elongate in a particular direction, and the images for iPSCs on the spin coated samples revealed cytoskeletons that were spread evenly.

The cytoskeleton of a cell went a particular direction, but it did not necessarily match up with that of the other cells. In another research study, it was revealed that using aligned electrospun fibers of the elastomer PLGA had the correct topography to allow murine myoblasts to elongate and align to form skeletal muscle [5]. Only the effects of the aligned fibers were studied as the cause of changes in cell adhesion, elongation, and differentiation of the myoblasts [5]. The data collected in this research is consistent with this previous study for elongation and cell adhesion, but different in terms of differentiation. Unlike the murine myoblasts, the iPSCs reverted to a less differentiated state, as indicated by an increase in stemness gene expression.

The gene expression levels for *NANOG* and *PECAM-1* were different among groups. Unlike previously hypothesized, the cells did not differentiate, but rather became more stem cell –like. The aligned and unaligned fibers were not significantly different in gene expression levels for the three genes, but electrospun samples significantly up-regulated gene expression compared to the spin coated samples, excluding the 90%PCL – 10%cPCL. An important discovery made during this project showed that using different components of the polymer stimulated major differences in iPSC behavior. A definite trend observed was that the spin coated samples of 90%PCL – 10%cPCL exhibited more gene expression that the other two combinatorial polymer. The spin coated sample of 4%PEG – 96%PCL had higher gene expression levels than the 100% PCL. The difference shown suggests that polymer composition has significant impacts on the stemness of iPSCs. Specifically, a property of the subunit cPCL seemed to cause iPSCs to express very high levels of the stemness markers. The bright field image of cells cultured on 90%PCL – 10%cPCL showed that very few cells remained adhered. This was not expected as cPCL is known to promote cell adhesion for other types of cells such as murine ESCs in Gupta, *et.al* [13].

CONCLUSION.

In this research, iPSCs were cultured on aligned and unaligned electrospun

combinatorial polymer fibers, spin coated polymer films, and glass. The question of how polymer composition and alignment affect stem cell differentiation was addressed through incorporating gene expression tests in this research. Interestingly, it was observed that the iPSCs became more stem-like when cultured on polymers containing various subunits, especially the composition containing the cPCL subunit. This finding is supported by the three different stemness markers that resulted in high expression. The research was significant as iPSCs had never been cultured on aligned electrospun combinatorial polymer fibers. Additionally, this project provides an investigation into the genotypic differences of iPSCs grown within these varying microenvironments with significant data.

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SUPPORTING INFORMATION.

Supplemental Methods and Results.

Figure S1. Setup for Aligned Fibers: A plastic square with a circle in the center had two grounded razor blades parallel to each other. Two cover glasses were placed between razor blades and so that aligned fibers would collect onto the cover glasses.

Figure S2. Analysis with the SEM images of the aligned samples showing the average angles, thicknesses, and spacings of the combinatorial polymers.

Figure S3. Brightfield Images: The images show the iPSCs cultured on the various scaffolds at day 4. The alignment and density of the cells can be observed.

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