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INVESTIGATION OF MSC DIFFERENTIATION ON ELECTROSPUN NANOFIBROUS SCAFFOLDS

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Emily Wible (Bioengineering) – University of Pennsylvania
Advisor: Robert L. Mauck and Nandan L. Nerurkar

ABSTRACT

Tissue engineering has been utilizing electrospun nanofibrous scaffolds as a platform for growing mesenchymal stem cells (MSCs). Electrospinning allows for the fabrication of scaffolds with a controllable degree of fiber alignment. Previous work has established that variation in the degree of fiber alignment directly effects the shape of the cells seeded onto these scaffolds. This study explored the effect of scaffold fiber alignment, and hence cell shape, on MSC differentiation. Two groups were observed in this study—aligned and non-aligned scaffolds. The scaffolds were seeded with MSCs and grown in a chemically defined culture medium for ten days. The effect of alignment was inspected using fluorescent microscopy to visualize cell morphology, biochemical assays to measure DNA content and glycosaminoglycan production, and real time PCR to determine gene expression. It was hypothesized that aligned scaffolds, in which cells are elongated in shape, would result in an up regulation of collagen type I, a marker of fibrous tissue differentiation, while non-aligned scaffolds would induce an up regulation of collagen type II, a cartilaginous marker. The data supported the second part of our hypothesis, that collagen type II would be up regulated in non-aligned scaffolds. However, collagen type I was not different between the two scaffold types. Because collagen type I is not unique to fibrous tissues, more specific markers are currently being investigated, including tenomodulin and lysyl oxidase. In conclusion, scaffold disorganization promoted chondrogenic differentiation of MSCs when compared to highly aligned fibers. These results support the contention that cell shape plays an important role in the differentiation of MSCs along fibro-cartilagenous phenotypes.

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1. INTRODUCTION

The long term goal of tissue engineering is to create functional tissue that replaces injured or degenerated native tissue. Currently, mesenchemyl stem cells (MSCs) have become a popular cell source due to their ability to differentiate into various cells, including chondrocytes, osteoblasts, and adipocytes. However, the mechanisms that dictate the differentiation process remain poorly understood. Therefore, it is necessary to explore the mechanisms that direct a cell towards a specific lineage.

Recently, nanofibrous electrospun scaffolds have become a possible platform for growing new tissue. Unfortunately, it is not well understood which scaffold features direct a cell towards a specific cell type. In previous research, it has been shown that cell shape is important in differentiation [1]. For example, cells residing in fibrous tissues have an elongated shape, while cartilaginous cells have a more rounded contour. In order to help encourage the MSCs on scaffolds to adopt a particular cell shape, fiber alignment has been manipulated to be either highly aligned or non-aligned. The hope is that aligned fibers will encourage the MSCs to adopt a fibrous phenotype, while the non-aligned fibers will encourage the MSCs to adopt a chondrogenic phenotype. This research study focuses on scaffold fiber orientation as a possible means to direct MSCs towards a specific phenotype.

2. BACKGROUND

MSCs are able to differentiate into various cells including chondrocytes, osteoblasts, and adipocytes. This variability makes MSCs an important resource for generating new tissues. However, it is important to determine the environmental conditions which direct MSCs towards a particular cell type. In order to assess the effect of an environmental condition, the expression of tissue specific genes can be used to indicate the phenotype of differentiated cells. SOX 9, a transcription factor, and collagen type II, an extracellular matrix protein, are both present primarily in chondrocytes, and therefore serve as markers of chondrogenic differentiation. In comparison, collagen I is more common in fibrous tissues such as tendon and ligament, and is therefore a marker of fibrous differentiation.

The MSCs in this research project were grown on electrospun nanofibrous scaffolds. Electrospun scaffolds have become an important area of research due to their ability to have an aligned or non-aligned fiber arrangement [2]. Aligned scaffolds have fibers oriented parallel to each other, while non-aligned scaffolds have fibers oriented randomly. Previous work has shown that cells grow along the fiber orientation [4]. This study hypothesized that aligned scaffolds, in which cells are elongated in shape, would result in an up regulation of collagen type I, a marker of fibrous tissue differentiation, while non-aligned scaffolds would induce an up regulation of collagen type II and SOX9, both cartilaginous markers.

3. METHODS

3.1 CELL CULTURE

Bovine MSCs were seeded on nanofibrous electronspun scaffolds for 10 days. Initially, bovine marrow was harvested from the femur and tibia. This marrow was then placed in tissue culture plastic dishes, containing basal media to allow the MSCs to crawl out. Once the MSCs were expanded to passage 2, they were seeded on the aligned and nonaligned electrospun scaffolds. From this point on, the MSCs were grown in a chemically defined growth media containing TGF-β3. At 3, 7, and 10 days of culture, real time PCR, actin staining, and biochemical assays were performed. For each time point, an extra group was added that was treated with cytochalasin. Cytochalasin depolymerizes actin, the primary cytoskeletal element of cells which gives rise to and maintains cell shape.

3.2 FLOURESENCE MICROSCOPY

Fluorescence microscopy was used to visualize both the nucleus and stress fibers of the cells on the scaffolds. The stress fibers provided not only a rough outline of the cell shape, but also an indication of whether the MSCs were growing along the fiber alignment. Samples were stained with phalloidin conjugated alexafluor to visualize the actin cytoskeleton, and counterstained with DAPI to visualize cell nuclei. Blue light was used to visualize the actin, while UV light was used to visualize the nucleus of the cells. Pictures were collected from both types of light at 20x and then overlaid into a single picture displaying both the nucleus and actin.

3.3 REAL TIME PCR (POLYMERASE CHAIN REACTION)

Real time PCR was used to simultaneously amplify and quantify a specified DNA product (determined by the genetic markers). The DNA content was quantified based on the intensity of fluorescence. SYBR Green, a DNA-binding dye, attached to any double-stranded DNA, causing the dye to fluoresce. As the amount of double-stranded DNA increased, so did the fluorescence. In order to make the results more accurate, a house-keeping gene was used in comparison to the gene of interest. The challenge with SYBR Green is that it is non-specific and will attach to all double-stranded DNA. Therefore, RNA samples were treated with DNAse prior to amplification to prevent genomic contamination. The genes observed in this study were SOX9, collagen type I, collagen type II, and GAPDH (house-keeping gene).

3.4 BIOCHEMICAL ASSAYS

Two different biochemical assays were used in this study. The first biochemical assay determined the amount of GAG in the scaffolds. Before the scaffolds were analyzed for GAG content, the scaffolds had to undergo a papain digest for 24 hours in a 60°C water bath. The papain digest consisted of 20 µl of papain per ml of papain digest buffer. After digestion, the scaffolds were analyzed for GAG content using 1,9-dimethylmethylene blue dye-binding (DMMB) assay. Standard samples of known concentrations of chondroitin-6-sulfate were run with the scaffold samples in order to calibrate the measurements. The presence of GAG served as a measure of chondrogenic differentiation, as GAG is present in high quantities in cartilaginous tissues.

The second biochemical assay determined the amount of DNA present in the scaffolds. Before the scaffolds were analyzed for DNA content, they had to undergo a

papain digest as described above. After digestion, the scaffold samples were analyzed for DNA content using the Picogreen ds-DNA assay. Standard samples of known concentrations were run with the scaffold in order to calibrate the measurements. The presence of DNA allowed for the determination of GAG/DNA so that GAG production could be analyzed without being obscured by varying cell quantities.

4. RESULTS

Figure 1, 2, and 3 display the ratio of the specified genetic marker (COLI, COLII, or SOX9) to the house-keeping gene (GAPDH). The house-keeping gene was used to normalize the results and account for varying cell contents. Significance in this study was determined by performing a two-tailed t-test with an alpha=0.05. Although there is no significant difference at day 10 in Figure 1, collagen type II is indeed significantly higher at day 10 in non-aligned scaffolds. SOX9, a transcription factor known to up regulate collagen type II expression was observed to confirm our finding that chondrogenesis is enhanced on nonaligned scaffolds. As shown on Figure 3, the collagen type II up regulation at day 10 coincided with up regulation of SOX9. It should be noted that the non-aligned group for day 10 only had a sample size of n=1.

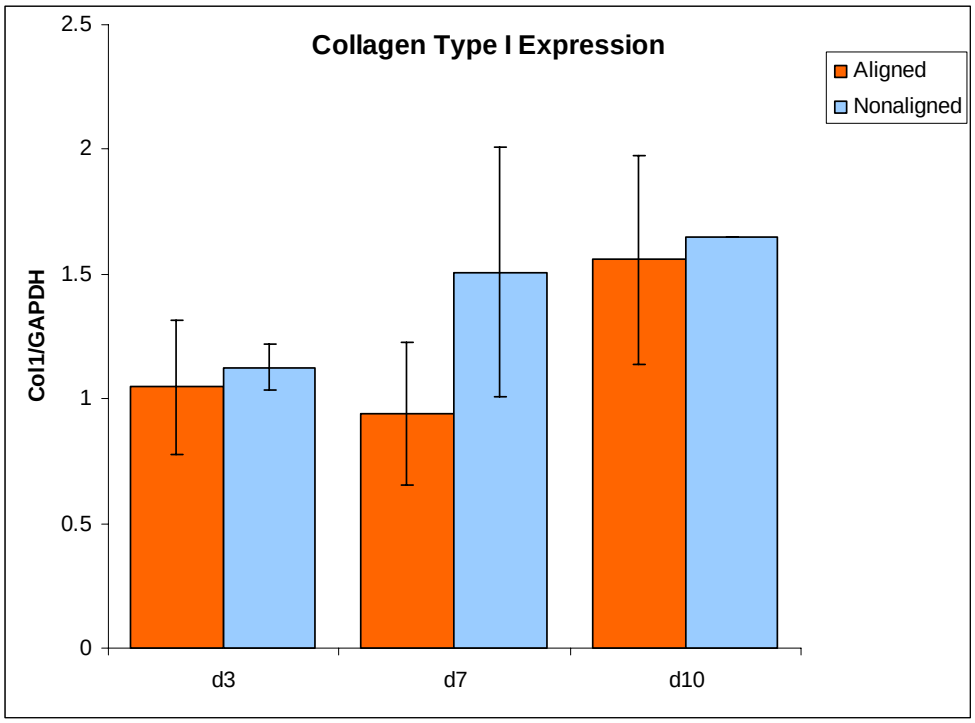

Figure 1.

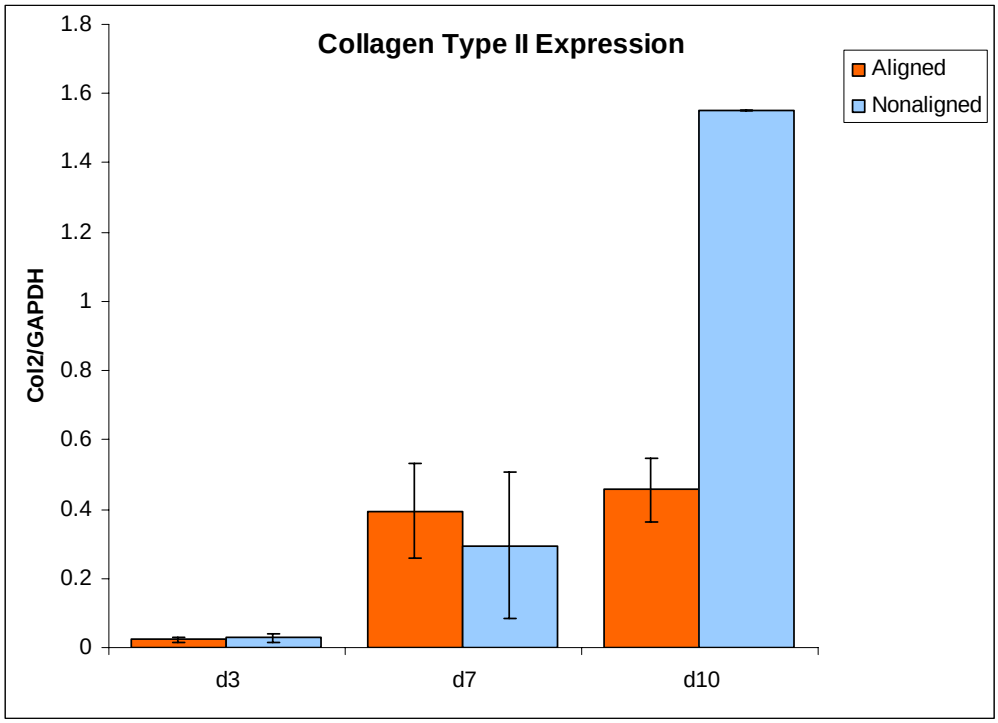
Collagen Type II Expression
Aligned
Nonaligned
Col2/GAPDH
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
d3
d7
d10

Figure 2.

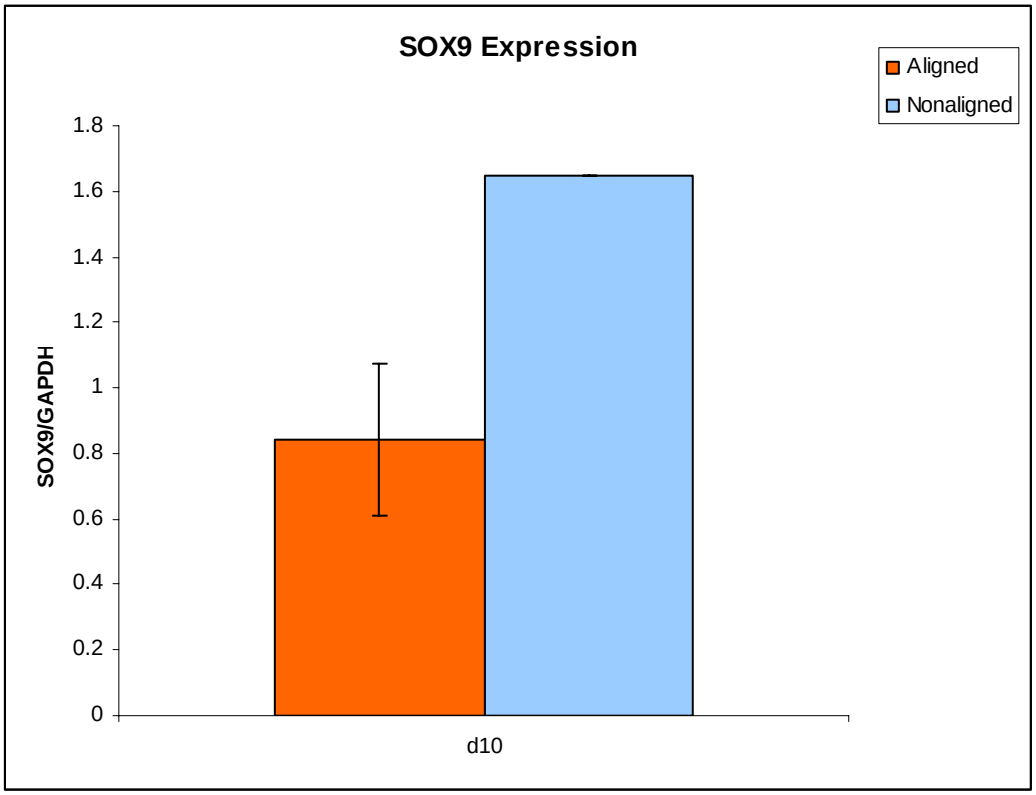

Figure 3.

Figure 4 is a picture of the cytochalasin treated groups (aligned and non-aligned) at day 10. The stress fibers have been disassembled and the green is seen scattered throughout the image, with blue nuclei indicating the presence of a cell.

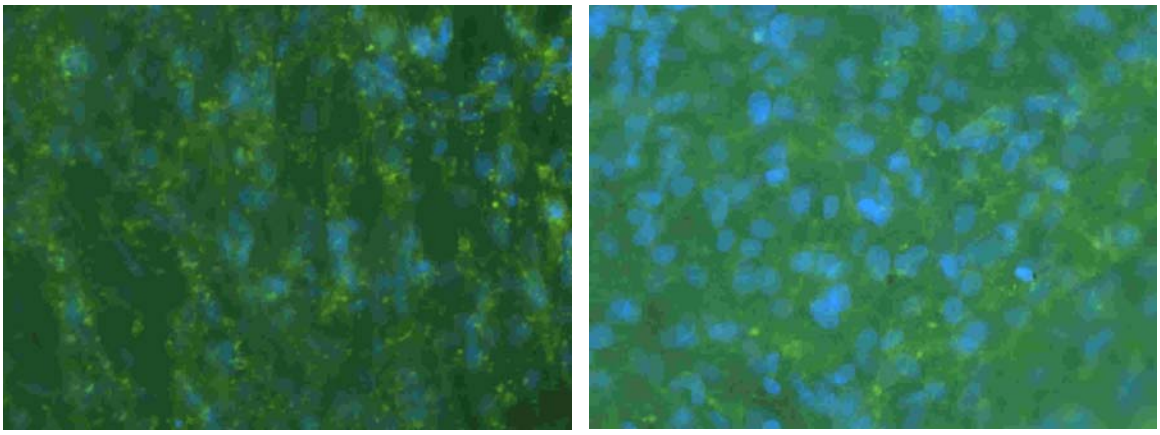

Figure 4.

Figure 5 displays the fluorescence microscopy of the non-aligned (bottom row) and aligned (top row) scaffolds for day 3, 7, and 10.

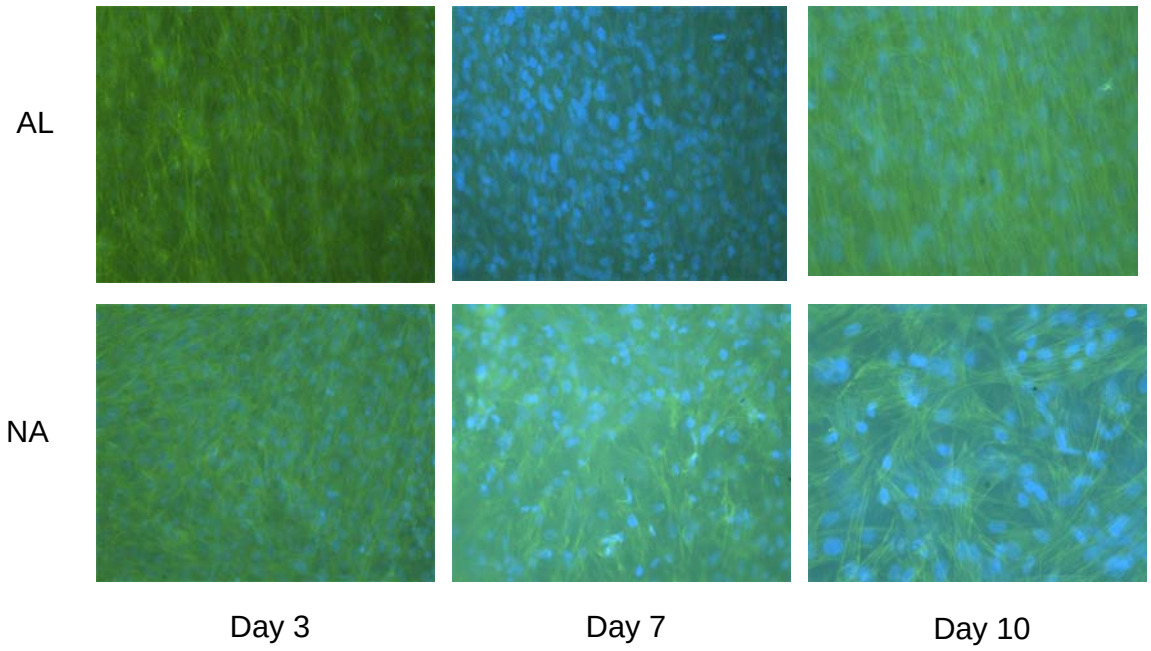

Figure 5.

The biochemical assay results are not depicted in the final paper; however, there were no significant differences observed between aligned and nonaligned scaffolds. There was, however, a consistent reduction of GAG production on both scaffold types when the scaffolds were treated with cytochalasin.

5. DISCUSSION AND CONCLUSIONS

As indicated by Figures 2 and 3, we observed an up regulation of SOX9 and collagen type II on the non-aligned scaffolds when compared to that observed on the aligned scaffolds. This partially supported our hypothesis that non-aligned scaffolds would encourage cells toward a cartilaginous type. The cells were encouraged to become cartilaginous because of the non-aligned fibers, mimicking a more rounded shape. Hence, shape plays an important part in directing the phenotype of a differentiated MSC.

On the contrary, as indicated in Figure 1, we did not observe any change in the presence of collagen type I on the aligned scaffolds when compared to the non-aligned scaffolds. Although the bar graph shows a difference on d7, this difference becomes insignificant at time point day 10 (tested with two-tailed t-test). Because collagen type I is expressed in undifferentiated MSCs, as well as chondrogenically differentiated MSCs, it may not be a good marker for fibrous differentiation. Currently, other markers are being investigated that might better confirm a fibrous cell's phenotype.

Figure 4 confirms that cytochalasin interrupts the actin filaments in the cell. After the actin has been disrupted, the cell no longer displays its original shape. The stress fibers are not as visible as they normally are on the scaffolds. These observations coincided with a loss of GAG production, and with a reduction in type II collagen expression.

Actin staining (Figure 5) visually showed the elongated shape of MSCs on aligned scaffolds and the rounded shape of MSCs on non-aligned scaffolds. This was seen for all time points (days 3, 7, and 10). These morphologies were outlined by the stress fibers' florescence in green and the nuclei's fluorescence in blue. The actin

staining also confirmed that the MSCs were growing along the fiber direction (aligned or non-aligned).

6. RECOMMENDATIONS

Currently, more specific genes for fibrous differentiation are being investigated to better indicate a fibrous phenotype. The two genes being investigated currently are tenomodulin and lysl oxidase. These genes are being considered because they are up regulated in fibrous tissue.

7. ACKNOWLEDGMENTS

I would first like to thank the National Science Foundation for their support through an NSF-REU grant and Microsoft Corporation for their financial support. I would also like to thank my advisor, Robert L. Mauck, for selecting me as a summer research student in his lab. I would finally like to thank my graduate student, Nandan L. Nerurkar, for all of his assistance with my summer project.

8. REFERENCES

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