Vesicle Trafficking as a Mechanism to Sense and Respond to Nanofiber Architecture

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Statement of Purpose: Procedures involving bone grafting are performed on over 1.5 million patients and at a combined cost of more than $2.5 billion annually. The majority of these procedures use autografts or allografts; however neither is an ideal material as autografts require a second surgery that often leads to donor site morbidity and allografts potentially generate an immune response and are capable to transmitting viral disease. Synthetic scaffolds do not pose such risks, but they lack the osteoinductive response necessary for bone replacement. Studies have shown that cells sense surface architecture and are capable of bending their membranes to conform to a curved surface. *Vogel and Sheetz* have hypothesized that arfaptin, a vesicle transport protein, may have an either/or relationship between binding a GTPase, such as Rac1, or a curved membrane. Theoretically, if the cell membrane is curved around a sufficiently small structure—on the order of magnitude of a vesicle—arfaptin will preferentially bind to the membrane and release Rac1. More unbound Rac1 should lead to more activated Rac1, which can then act to accelerate Rac1 mediated MAPK signaling, a signaling cascade responsible for cell proliferation and differentiation, leading to an osteoinductive response. Similarly, other vesicle binding proteins may also bind to curved cell membranes depending on the level of curvature. We hypothesize that vesicle trafficking proteins are geometry sensors capable of binding unique fiber diameter ranges and that sufficiently small fibers will result in elevated levels of active Rac1.

Methods: Electrospinning was used to create poly (L-lactic acid), PLLA, nanofibers of varying diameters, with the target sizes being <100 nm, 200 nm, and 500 nm. Diameters were quantified using ESEM. MC3T3-E1 S4 cells were seeded onto nanofiber substrates and were immunostained to determine if arfaptin, Rac1, and active Rac1 localize along nanofibers. Staining for clathrin and caveolin was also performed to evaluate if other vesicle trafficking proteins localized along nanofibers. A modified immunoprecipitation procedure was used to quantify the levels of total and active Rac1.

Results:
Extensive testing with numerous electrospinning solutions and conditions has resulted in nanofibers as small as 42 nm with 83 nanometers being the average diameter. The electrospinning conditions used to achieve this diameter range were 30G needle, 0.3 ml/hr, 10 cm, and 10 kV with a solution of 1% PLLA (MW 650 kDa) in 65% tetrahydrofuran: 30% pyridine. The fibers were uniform in size but did exhibit minor amounts of beading.

Cells were stained at six (not shown) and twenty-four hours (Figure 1) for both active and total Rac1. As seen in Figure 1, active Rac1 tends to localize along the width of the nanofiber (distinguished by the yellow box). No such localization was exhibited in the control. These results were repeatable (n=6). Initial immunoprecipitations failed to yield usable results. Preliminary results with other vesicle trafficking proteins on larger nanofibers (200-500 nm) indicated that clathrin does localize along nanofibers (Figure 2).

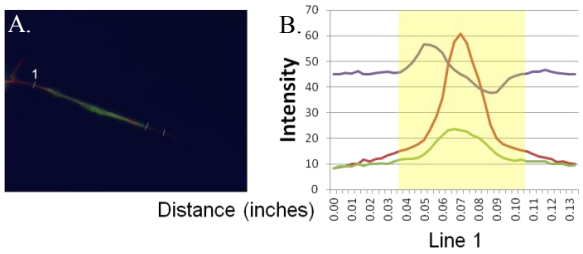

Figure 1: RGB image of a cell aligned on a nanofiber (A) and line graph of line 1 in the image (B). Red channel is active Rac1, green channel is total Rac1, and the blue channel is the DIC surface image. The yellow box represents the approximate edges of the cell. This data indicates that active Rac1 localizes along the nanofibers.

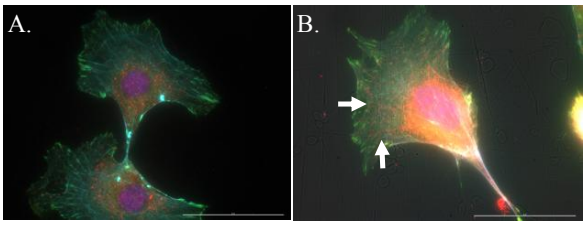

Figure 2: Cells adhered to PLLA control (A) and nanofibers (B). Immunostaining coloring is purple-DAPI, blue-actin, green-vinculin, and red-clathrin. Clathrin localization indicated with arrows. Scale bar is 50 μm.

Conclusions:
Active Rac1 has been shown to localize along nanofibers of <100 nm in diameter, giving strength to Vogel's hypothesis that nanofiber diameter is a means to control intracellular signaling via vesicle trafficking proteins. These proteins, specifically clathrin, were shown to localize along the fibers. Further testing with smaller nanofibers may demonstrate localization of the other vesicle proteins to the nanofibers. The active and total Rac 1 immunoprecipitation protocol needs to be optimized to attain quantifiable results. With these quantitative measurements of Rac1 levels, Vogel's hypothesis may be fully supported.

References: Vogel V, Sheetz M. Local force and geometry sensing regulate cell functions. *Nature reviews. Molecular cell biology*. 2006;7(4):265-75.