Nanofiber mediated osteoinduction through RhoA GTPase signaling

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Statement of Purpose: The availability of an adhesive surface to a cell modulates the cellular response in different ways. One interesting way to manipulate adhesive area is to alter the topographical cues (others possibilites are surface ligand density and stiffness of the surface). Previous studies showed that surface topographies play critical roles in differentiating osteoblasts independent of chemical factors (1). While adhering to certain topographies, osteoblats undergo drastic morphological changes including enhanced focal adhesions, stress fiber formation and increased contractility (upon formation of acto-myosin complexes). RhoA GTPase signaling cascade is activated upon cytoskeletal changes and often leads to downstream signaling cascades closely associated with cellular growth and differentiation. This study investigates whether changes in surface topography, especially extracellular matrix like fibrous topography, would lead to differentiation of osteoblast cells through RhoA GTPase induced contractility and further lead to changes in growth/differentiation signals.

Methods: Poly(methylmethacrylate) (PMMA) nanofibers were produced using electrospinning. The average diameters of fibers were measured to be 850 nm. As a material control 2% PMMA solution was spin coated onto a glass cover slip. MC3T3/E1 cells were seeded on either flat PMMA coated or fiber coated cover slips. The cell morphology was observed using immunofluorescence imaging. A modified probe Atomic Force Microscopy (AFM) was used to measure the mechanical properties of the cells growing on fibrous and flat PMMA substrates. In this method the cellular stiffening due to cytoskeleton rearrangement results in viscosity changes which were measured by the amount of deflection of the cantilever tip that translates the force applied by the substrates into spring deflection (Fig 2. C). Following the previously published modified Hertz model [4];

$$\delta = 3F(1-v2)4ER2/3$$

where δ is the deformation of the sample, F is the applied force, v is the Poisson's ratio (0.5), E is the elastic modulus and R is the radius of the sphere. In order to investigate the cellular growth and differentiation signaling ERK1/2, p38 and JNK/SAPK semi-quantitative western blot was performed with inhibitors of cytokeletal tension related to RhoA GTPase signaling. We also examined directly RhoA GTPAse activity using a RhoA activity assay (Cytoskeleton Inc). In order to test osteogenic differentiation ALP staining was performed using SigmaKit No85 after 2 week incubation on experimental substrates.

Results: Previous studies from our group show that (2) growth and differentiation signals of MAPK signaling cascade change significantly when fibers diameters reach 800 nm. We hypothesized that the observed changes could be due to increased cytoskeletal stiffness along a fiber which an individual cell is adhered to. We first measured the cell stiffness and found a cell on a fiber was three times stiffer than a cell on a flat surface. We further investigated whether the stiffness is due to RhoA→ROCK (Rho associated coiled coil kinase) →MLCK pathway. When a ROCK inhibitor was added the stiffness dropped to 2.75 MPa approximately 40 percent less than a cell on a flat substrate and 5 times less than a cell on a fiber (Table 1).

	Cells on PMMA	Cells on glass slide	Cells on fibers	Cells on fibers with Y27632
Mean	5.62 MPa	7.13 MPa	15.3 MPa	2.75 MPa
Std Dev.	3.62 MPa	1.92 MPa	5.89 MPa	1.39 MPa

Table 1.The stiffness values of individual cells adhering to flat PMMA or fiber coated surfaces.

The observed changes in stiffness due to RhoA could potentially propagate to changes in conserved signaling cascades. We further tested if RhoA mediated stiffness contributes to p38 signaling activity which has been reported to be vital to osteoblast growth and differentiation (2). Figure 1 shows the p38 activity after addition of ROCK inhibitor Y27 and myosinIIa inhibitor Blebbistatin. While cells on flat PMMA increase their p38 activity by 40% in response to blebbistatin, p38 activity of a cell on a fibrous substrate supressed until 4 hour time point then had a 50% increase.
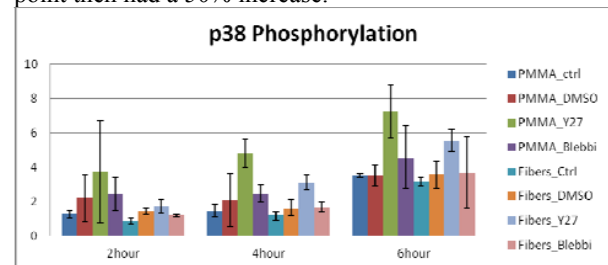

Figure 1. Relative changes in p38 phosphorylation in response to ROCK inhibitior (Y27) and MyosinIIa inhibitors on flat (PMMA) vs fiber coated surfaces.

Conclusions: RhoA involvement in initial adhesion to extracellular matrix like topographies have significant effects on growth and differentiation signals on primary osteoblast cells.

References:

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2. Jaiswal D, Brown JL. Nanofiber diameter-dependent MAPK activity in osteoblasts. J Biomed Mater Res A. 2012 Jun 14.