Determination of significant kinase and phosphatase genes on tissue engineered osteoinduction using a novel high-throughput siRNA screening strategy

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Statement of Purpose: Biomaterials play significant roles in regenerative therapies and various studies reported significantly increased bone regeneration with fibrous scaffolds (1). The biomimetic nature of fibrous scaffolds has previously demonstrated an increase in the production of various bone specific extracellular matrix proteins as well as inorganic molecules such as Hydroxyapetite (HA), calcium triphosphate ($Ca_3(PO_4)_2$) and composite crystals of both HA and ($Ca_3(PO_4)_2$). However the use of biomaterials for regenerative therapies is a relatively new field and detailed investigation is required in order to elucidate the molecular interactions that take place at the cell biomaterial interface. Several studies have attempted to explain the global overview of key signaling components that take place in bone regeneration on biomaterials scaffolds, however no one has yet developed a global model of signaling molecules that drives osteoinduction on synthetic bone regenerative engineering scaffolds.

Methods: In this study we tested the effect of substrate topography, in particular the curvature that is provided by synthetic polymer fibrous scaffolds. We used 2 siRNA libraries (a Phosphatase Library that consists of 237 genes and a Kinase library that consists of 636, a total of 873 genes) to study the osteogenic phenotype observed on flat PMMA and PMMA fiber scaffolds. In order to perform high throughput screening experiments a special 96 plate was developed and optimized for assay conditions.

As phenotype output for the assay we used two experiments. The first experiment, Alamar Blue, is used to detect the change in the cell metabolism as a consequence of sirna delivery and LOF. We used Alamar Blue to filter out the genes with a low score (an indicator of low cell viability). The second experiment is high throughput In-Cell Western Blot for an early osteoblast marker transcription factor, RUNX-2 (cbf**α**-1), in the human osteoblast like cell line SaOS-2. After a series of filtering and organizing, the genes that are significantly up-regulated (UR), downregulated (DR) or did not change (NC) (Table 1) RUNX-2 levels were further enriched using INGENUITY gene enrichment analysis software. The results shed light into molecular mechanisms that specifically play a role on substrate curvature mediated osteoinduction on synthetic regenerative engineering scaffols.

Table 1. Summary of upregulating (UR), downregulating (DR) and not changing (NC) genes

	Topography					
	PMMA			Fibers		
Library	UR	NC	DR	UR	NC	DR
Kinase	104	52	104	120	60	120
Phosphatase	42	21	42	46	23	46

Results: Once the data normalization was complete the samples were binned into upregulating, downregulating and not changing bins. Since the independent sample distributions deomonstrated slight differences and unbiased selection criteria was used for hit selection. Once the density distributions were obtained we picked the top 40% of the high tail of the data for the upregulating gene bin, the lower 40% for the downregulating gene bin and the middle 20% for the not changing gene bin. This ensures a global selection was met after all sample values were normalized to Runx2 and Alamar blue values according (Table 1).

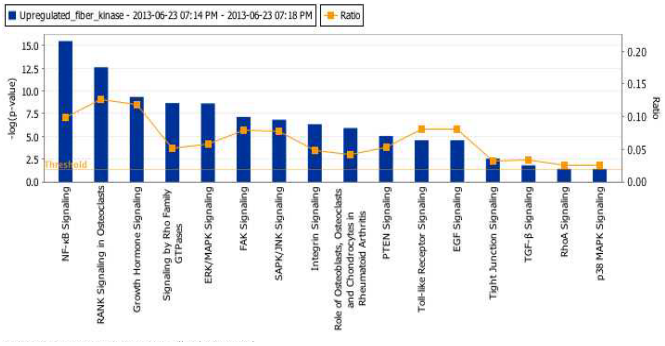

Figure 1. Significant Canonical Pathways effected by substrate topography.

Conclusions: The interactions between the significant genes found in our study and the whole signaling cascade could be traced and the possible up or down stream targets can be obtained from the maps obtained via IPA. IPA results suggested that there were 58 proteins associated with cellular signaling mechanisms, 48 with tissue morphology, 44 with small molecule biochemistry such as growth factor signaling and 73 proteins activated that correlated with cell proliferation and growth. IPA also allowed us to analyze highly active canonical pathways and NF-Kappa B signaling demonstrated the smallest p values of $2.16* 10^{-17}$ with 14 of the 83 signaling components active in the siRNA results. Figure 1 also represents the active components in canonical NFKappa B and integrin signaling cascade.

References:

1. T. Ozdemir, L. C. Xu, C. Siedlecki and J. L. Brown, *Integr Biol (Camb)*, 2013, **5**, 1407-1416