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Short communication

Mapping the osteocytic cell response to fluid flow using RNA-Seq

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ABSTRACT

Bone adaptation to mechanical loading is regulated via signal transduction by mechano-sensing osteocytes. Mineral-embedded osteocytes experience strain-induced interstitial fluid flow and fluid shear stress, and broad shifts in gene expression are key components in the signaling pathways that regulate bone turnover. RNA sequencing analysis, or RNA-Seq, enables more complete characterization of mechano-responsive transcriptome regulation than previously possible. We hypothesized that RNA-Seq of osteocytic MLO-Y4 cells reveals both expected and novel gene transcript regulation in cells previously fluid flowed and analyzed using gene microarrays. MLO-Y4 cells were flowed for 2 h with 1 Pa oscillating fluid shear stress and post-incubated 2 h. RNA-Seq of original samples detected 55 fluid flow-regulated gene transcripts (p-corrected <0.05), the same number previously detected by microarray. However, RNA-Seq demonstrated greater dynamic range, with all 55 transcripts increased >1.5-fold or decreased <0.67-fold whereas 10 of 55 met this cut-off by microarray. Analyses were complimentary in patterns of regulation, though only 6 transcripts were significant in both RNA-Seq and microarray analyses: *Cxcl5*, *Cxcl1*, *Zc3h12a*, *Ereg*, *Slc2a1*, and *Egln1*. As part of a broad inflammatory response inferred by gene ontology analyses, we again observed greatest up-regulation of inflammatory C-X-C motif chemokines, and newly implicated HIF-1α and AMPK signaling pathways. Importantly, we detected both expected fluid flow-sensitive transcripts (e.g. *Nos2* [iNOS], *Ptgs2* [COX-2], *Ccl7*) and transcripts not previously identified as flow-sensitive, e.g. *Ccl2*. We found RNA-Seq advantageous over microarrays because of its greater dynamic range and ability to analyze unbiased estimation of gene expression, informing our understanding of osteocyte signaling.

1. Introduction

Mechanical signals profoundly influence cellular behavior. In response to external stimuli such as fluid shear stress, cells experience membrane deformation, conformational changes in transmembrane proteins, and dynamic cytoskeletal restructuring leading to fluctuations in gene expression. These mechano-sensitive genes compose a complex web of signaling pathways that remains poorly understood. Characterizing mechanical regulation of these genes and their signaling roles may enable control of cellular responses to mechanical signals.

Matrix-embedded osteocytes, the putative mechano-sensors of bone, are subject to interstitial fluid flow upon whole bone loading (Bonewald, 2011; Klein-Nulend et al., 2015; Schaffler et al., 2014). Osteocytic cells exposed to such fluid shear stress in turn up-regulate differentiation of bone-forming osteoblastic progenitors by undetermined cell-to-cell and paracrine signaling mechanisms (Taylor et al., 2007), counterbalancing osteoclastic mineral resorption. We previously characterized the global response of osteocytic MLO-Y4 cells to fluid flow using hybridization-based microarray analysis of gene transcripts (Govey et al., 2014). We identified regulation of both expected and novel gene transcripts in response to flow and corroborated findings with qPCR.

However, next generation sequencing technology provides new advantages in directly quantifying the transcriptome via RNA sequencing, or RNA-Seq. We hypothesized that RNA-Seq—not previously applied to fluid flow mechanotransduction—reveals additional insights into osteocytic signaling. By analyzing the same samples, we were able to directly compare results from RNA-Seq and microarray analysis, discuss merits of each technique, and conclude that RNA-Seq adds valuable insight not apparent from microarrays. Furthermore, we report regulation of additional fluid flow-sensitive gene transcripts, notably *Nos2, Ptgs2*, and inflammatory chemokines

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Cxcl3, *Ccl2*, and *Ccl7*, as well as gene ontology (GO) analysis of inferred signaling pathways.

2. Methods and methods

2.1. Fluid flowed osteocytic MLO-Y4 cells

To allow direct comparison to previous results, we analyzed the same total RNA, stored at −80 °C, as analyzed in our previous study (Govey et al., 2014). RNA quality was verified using an Agilent 2100 Bioanalyzer. Samples were originally treated as follows (Fig. 1A): osteocytic MLO-Y4 cells were cultured on collagen I-coated glass slides for 2 days and then exposed to 2 h of sinusoidally oscillating fluid flow in parallel plate flow chambers, inducing 1 Pa (10 dynes/cm^2) shear stress at 1 Hz. Paired sham controls were maintained in identical, static chambers. Triplicates of both flowed and static cells were collected immediately (0 h post-flow) and following 2, 8, and 24 h incubation in fresh medium (α-MEM [Invitrogen, Grand Island, NY] with 2.5% calf serum [Hyclone, Logan, UT], 2.5% fetal bovine serum [Lonza, Walkersville, MD], 1% Penicillin/Streptomycin). Total RNA was isolated using Qiagen Rneasy Mini Kits. As the greatest number of transcriptional changes were previously found at 2 h post-flow incubation, we deep sequenced these samples.

2.2. RNA-Seq workflow

High quality RNA was subjected to a cDNA library preparation using the NEXTflex™ Rapid RNA Sequencing Kit (BioO Scientific, cat#:5138-02) and NEXTflex™ RNA-Seq Barcodes (BioO Scientific, cat#:512913), followed by Illumina sequencing using a HiSeq 2500 that was set to run for 50 cycles using a single-read recipe (TruSeq SBS Kit v3) according to the manufacturer's instructions. Adapter trimmed and quality filtered sequencing reads were aligned to the mouse reference genome build mm10 using Tophat v2.0.9 (Trapnell et al., 2009). Normalized gene expression values (FPKM, Fragments Per Kilobase of exon per Million fragments mapped) were calculated by Cufflinks v2.0.2 (Trapnell et al., 2010) provided with the gene annotation file available at Illumina's iGenomes (http://support.illumina.com/sequencing/sequencing_software/igenome.html). Differential gene expression was analyzed in Cuffdiff, which computed Benjamini–Hochberg false discovery rate correction. A corrected p-value < 0.05 was considered significant in this workflow (Fig. 1B). Gene transcript fold-changes indicative of flow regulation were calculated as (average flow)/(average non-flow). We visually confirmed exon coverage of all transcripts increased by >2-fold or decreased <0.5-fold (Fig. 3) using the UCSC Genome Browser (Kent et al., 2002) as in Fig. 1C. Complete RNA sequencing data was submitted to the Gene Expression Omnibus under accession record GSE70667.

2.3. Gene ontology (GO) functional associations

A list of 55 gene transcripts satisfying $p < 0.05$, all >1.5-fold change cut-off, was input to Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) for collective analysis of inferred signaling pathways and functions. Significance of the association between this dataset and canonical pathways was measured by (1) a ratio of the number of molecules from our data set that map to the pathway and (2) a p-value calculated by Fisher's exact test, which determines the probability that each biological function is due to chance alone. A broader list of 88 transcripts meeting a $p < 0.1$ cut-off was input to Cytoscape with the ClueGO plug-in (Bindea et al., 2009) to visualize a network of functionally grouped gene ontology (GO) terms significantly associated at $p < 0.05$ or $p < 0.1$ with gene transcripts serving as edge-connected nodes. In both analyses, GO associations were curated for relevance to the bone context.

3. Results

3.1. Comparison of results from RNA-Seq and microarray

Approximately 10,000 gene products were detected and could be quantified—most without significant regulation—from among just over 39,000 gene products that the reference genome was capable of mapping (Fig. 2). We identified 55 significantly regulated gene transcripts ($p < 0.05$, Supplementary Table 1) with

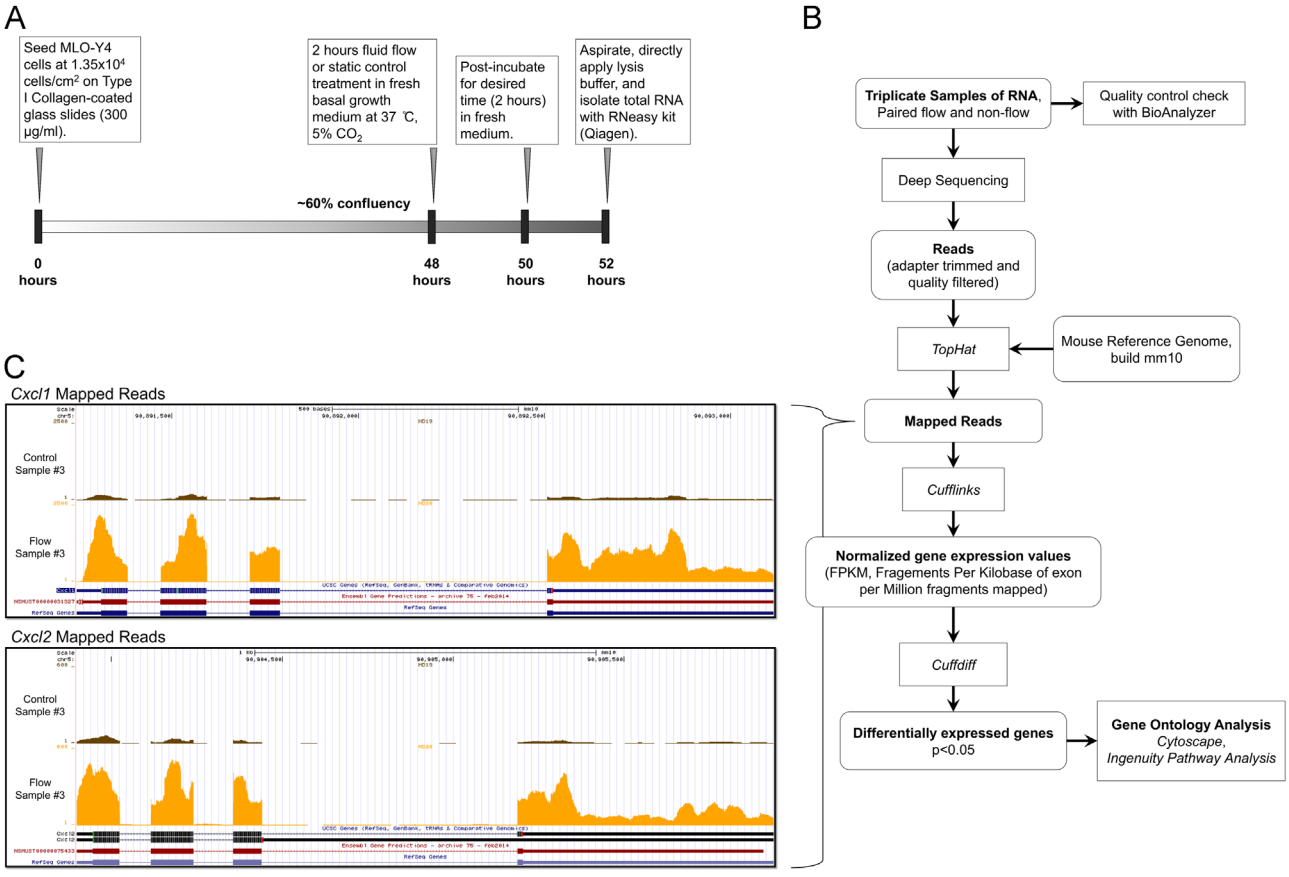

Fig. 1. (A) Experimental timeline for fluid flow treatment of osteocytic MLO-Y4 cells. (B) Workflow for RNA-Seq analysis using the Tuxedo Suite tools Tophat, Cufflinks, and Cuffdiff. (C) Representative bar graphs of reads vs. base pair from Control and Flow samples illustrate mapping to corresponding regions of the mouse genome encoding *Cxcl1* and *Cxcl2*, indicated by thicker bars (reference exons) below reads. Expression was visualized with the UCSC Genome Browser (http://genome.ucsc.edu).