Novel early response genes in osteoblasts exposed to dynamic fluid flow

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Cyclic mechanical loads applied to the skeleton from habitual physical activity result in increased bone formation. These loads lead to dynamic pressure gradients and oscillatory flow of bone interstitial fluid, which, in turn, exposes cells resident in the bony matrix to oscillatory fluid shear stress. Dynamic fluid flow has previously been shown to be a potent anabolic stimulus for cultured osteoblasts. In this study, we used cDNA microarrays to examine early phase, broad-spectrum gene expression in MC3T3-E1 osteoblasts in response to physical stimulation. RNA was harvested at 30 min and 1 h post-stimulation. RNA was used for microarray hybridization as well as subsequent reverse transcription polymerase chain reaction (RT-PCR) validation of expression levels for selected genes. Microarray results were analysed by both functional and expression profile clustering. We identified a small number of genes at both the 30 min and 1 h timepoints that were either upregulated or downregulated with flow compared to no-flow control by twofold or more. From the group of genes upregulated at 30 min, we selected nine for RT-PCR confirmation. All were found to be upregulated by at least twofold. We identify a novel set of early response genes potentially involved in mediating the anabolic response of MC3T3 osteoblasts to flow, and provide functional groupings of these genes that may shed light on the relevant mechanosensory pathways involved.

Keywords: osteoblast; fluid shear stress; cellular mechanotransduction;
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1. Introduction

Mechanical loading of bone is known to be a potent osteogenic stimulus (Jones *et al.* 1977). A variety of cell-level biophysical signals occur due to this loading, including, but not limited to, direct cellular deformation, generation of bioelectric fields and interstitial fluid flow (Bloomfield 2001). *In vitro* cell culture experiments have suggested that dynamic fluid flow at levels and frequencies expected to occur due to loading is associated with numerous osteogenic effects (Jacobs *et al.* 1998; You *et al.* 2000, 2001, 2002; Donahue *et al.* 2001, 2003*a,b*; Saunders *et al.* 2001, 2003; Li *et al.* 2004; Kim *et al.* 2006), and may be responsible in part or in full for bone mechanosensitivity (Qin *et al.* 2003).

Interstitial fluid flow occurs in two types within bone matrix: unidirectional flow and oscillatory flow. Unidirectional flow can be thought of as constitutive in nature, secondary to the constant arterial pressure head of the bone marrow. In contrast, the fluid flow that occurs due to mechanical loading is dynamic and specifically oscillatory in nature. Because of its inducible nature in response to loading, this dynamic oscillatory flow is thought to be the critical component involved in loading-induced osteogenesis (Jacobs *et al.* 1998).

One effect of cyclic loading and dynamic fluid flow at the level of osteocytes and osteoblasts is cellular shear stress (Jacobs *et al.* 1998). Previously, the effects of dynamic flow on osteoblasts *in vitro* have been investigated through the measurement of several response variables, including increased intracellular calcium mobilization, increased mitogen-activated protein (MAP) kinase activity, increased osteopontin gene expression (You *et al.* 2001), as well as increased prostaglandin E₂ release (Saunders *et al.* 2001). Although many specifics of the cellular response activated by dynamic fluid flow have been documented, a complete picture of the signalling pathways and mechanisms responsible for translating the mechanical stimulus into intracellular responses has been difficult to obtain. In this study, we utilized a broad cDNA expression microarray approach to identify candidate novel early response genes potentially involved in the mechanosensory response of MC3T3-E1 cells to flow. The advantage of this approach is that it allows a large-scale determination of gene expression without the bias introduced by the requirement that the investigator selects a small subset of target genes to investigate. Furthermore, the large set of data that results can be examined by bioinformatics approaches that allow examination of biological systems rather than changes in individual genes that are more prone to artifactual or misleading responses (Sherlock *et al.* 2001; Yang *et al.* 2008; Boyle *et al.* 2009).

2. Material and methods

(a) Cell culture

MC3T3-E1 osteoblast cells were cultured in minimum essential media alpha (α MEM) supplemented with 10 per cent foetal bovine serum (FBS) and 1 per cent penicillin/streptomycin at 37°C in humidified air with 5 per cent CO₂. For fluid-flow experiments, cells were seeded onto uncoated glass slides (76 × 48 × 1 mm) at a density of 3 × 10⁵ cells per slide. Fluid-flow experiments were conducted 48 h after seeding cells onto slides, at which time cells had achieved 80–90 per cent confluency.

(b) Oscillatory fluid flow (OFF)

MC3T3-E1 cells were exposed to flow using a previously described feedback-controlled fluid-flow device (EnduraTec, Eden Prairie, MN; [Batra *et al.* 2005](#)). Slides with cells were loaded individually into custom-built sterile parallel-plate flow chambers. Dynamic flow was produced with a Hamilton glass syringe connected in series with rigid walled tubing. The syringe was mounted in and driven by a linear electromagnetic actuator capable of delivering a precise flow rate. Complete culture media was used as flow media. Flow rate was monitored with an ultrasonic flow meter (Transonic Systems Inc., Ithaca, NY), and the regime resulted in maximum shear stresses of ± 1 Pa using a sinusoidal profile at 1 Hz. The duration of flow was 1 h. Static control slides were loaded into parallel-plate flow chambers but not exposed to fluid flow. Both flow and no-flow control chambers remained in the incubator during the 1 h duration of the experiment. After 1 h, slides were removed from the flow chambers, transferred into polystyrene dishes containing 15 ml of fresh media and placed in the incubator for either 30 min ($N = 3$ slides for flow and no flow) or 1 h ($N = 4$ for flow and $N = 3$ for no flow), yielding two post-flow timepoints. The 1 h timepoints were selected because they had previously shown to be sufficient to observe early changes in osteogenic gene expression and the 30 min timepoints were selected to observe regulatory gene signalling networks that might induce these changes ([Donahue *et al.* 2003b](#); [Reilly *et al.* 2003](#); [Saunders *et al.* 2003](#); [Malone *et al.* 2007](#)).

(c) RNA isolation

Slides were rinsed with ice-cold phospho-buffered saline after either 30 min or 1 h of post-flow incubation. Total RNA was extracted from each slide using the Qiagen RNeasy kit (Qiagen, Valencia, CA), and RNA was quantitated and verified for purity using ultraviolet (UV) spectroscopy at 260 and 280 nm. Total RNA samples from individual slides were pooled within each group to yield sufficient RNA for cDNA microarray probe generation and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR).

(d) Linear RNA amplification

Pooled total RNA of 5 μ g from each group was linearly amplified (RiboAmp Kit, Arcturus Bioscience, Mountain View, CA) to generate antisense RNA (aRNA; [Wang *et al.* 2000](#)). Briefly, mRNA species contained within the total RNA sample were converted into double-stranded cDNA templates bearing a T7 RNA polymerase promoter. An *in vitro* transcription reaction was subsequently carried out to generate aRNA from double-stranded cDNA. Amplification resulted in 50–80 μ g aRNA for each group. aRNA quality was checked by agarose gel electrophoresis, and quantity and purity were also determined by UV spectroscopy at 260 and 280 nm.

(e) Microarray hybridization

aRNA of 5–7 μ g from each flow and no-flow group at both timepoints were used for microarray probe generation. aRNA was reverse transcribed using random hexamers into cDNA probes labelled with either Cy3 or Cy5 dye. cDNA microarrays (MM Array, Stanford Functional Genomics Facility,

Stanford, CA) representing approximately 18 200 mouse genes were hybridized in quadruplicate for both the 30 min and 1 h timepoints with fluor reversal in two of the replicate arrays to minimize dye-dependent bias. Probes were purified, applied to microarrays and hybridized for 16 h at 65°C. Arrays were then washed and scanned immediately after hybridization using the Genepix 4000B scanner (Molecular Devices, Sunnyvale, CA), and array images were analysed with GENEPIX PRO 5 software (Molecular Devices). The resulting ratio data for each spot on the arrays were uploaded into the Stanford Microarray Database website and normalized using a spatial two-dimensional algorithm with log-linear interpolated background correction (Edwards 2003). Genes showing upregulation or downregulation in flow versus no flow by at least twofold on three or more replicate arrays at either timepoint were selected for hierarchical clustering analysis. This criterion is relatively conservative, but ultimately somewhat arbitrary and based on the quality and consistency of the data, experience and the desired number of genes to select for further analysis (Yang *et al.* 2008). Initial hierarchical clustering of genes and arrays was performed with CLUSTER 3.0 software (Human Genome Center, University of Tokyo, Tokyo, Japan) using average linkage with Euclidean distance as the similarity metric.

(f) *Microarray profile clustering analysis*

Further clustering analysis based on expression profile was performed using the Multi Experiment Viewer (MEV; <http://www.tm4.org/mev.html>) program supplied by The Institute for Genomic Research (TIGR). A logarithm base 2 transformation was applied to the data to produce continuous values and to treat up and downregulated genes in a similar way. We used a K-median clustering algorithm to cluster gene profiles based on expression-pattern similarity. For this analysis, the 30 min and 1 h timepoints were treated as independent.

(g) *Microarray statistical and functional clustering analysis*

The microarray data were next subject to significance analysis of microarrays (SAM; <http://www-stat.stanford.edu/~tibs/SAM/>). This more rigorous statistical analysis allows permutation of the data for multiple testing hypotheses. For this analysis, genes with a high variance were first eliminated and results for each timepoint were pooled. A false discovery rate (FDR) of less than 1 per cent was used to select genes for subsequent functional analysis and generate a rank-ordered list by fold expression. The microarray functional grouping of upregulated and downregulated genes was then performed with the online Database for Annotation, Visualization and Integrated Discovery (DAVID; National Institute of Allergy and Infectious Diseases, Bethesda, MD; <http://apps1.niaid.nih.gov/david>; Dennis *et al.* 2003). This analysis results in GeneOntology (GO) categories that show high enrichment in our data.

(h) *Quantitative real-time reverse transcription polymerase chain reaction*

Nine genes that displayed significant upregulation in flow-exposed cells relative to no flow using microarray analysis at the 30 min timepoint were selected and further analysed using quantitative real-time RT-PCR (Perkin Elmer Prism 7900, Applied Biosystems, Foster City, CA). The nine selected genes were assayed at

both the 30 min and 1 h timepoints. The same pooled total RNA samples used for microarray probe generation were used as the starting material. Primers and probes were commercially obtained from Applied Biosystems (Taqman Gene Expression Assays, Applied Biosystems, Foster City, CA). Additionally, rRNA for the housekeeping gene 18S (Taqman Gene Expression Assays, Applied Biosystems, Foster City, CA) was analysed as a control for the polymerase chain reactions (PCRs). All nine genes of interest were normalized against 18S rRNA. Each RNA sample was analysed in triplicate.

3. Results

All raw microarray data for this study can be found at the National Center for Biotechnology Information Gene Expression Omnibus (Edgar *et al.* 2002; GEO Series accession number GSE16211). Analysis of these raw data revealed that the expression of a remarkably small set of genes was affected by exposure to dynamic fluid flow. At the 30 min timepoint, 103 known genes and expressed sequence tags (ESTs) were upregulated by twofold or more with flow compared to no flow, while 67 known genes and ESTs were downregulated by twofold or more. At the 1 h timepoint, 133 known genes and ESTs were upregulated in the flow group, while 53 were downregulated. Genes were considered to be upregulated or downregulated if they showed a twofold or greater change with flow relative to no flow in at least three of four replicate arrays for each timepoint. Only approximately 1 per cent of the 18 200 mouse genes included on the microarray were found to be altered at each timepoint. The specific genes and their fold regulation arranged in functional groups are included in the electronic supplementary material of this paper.

The expression profile clustering analysis resulted in four clusters with similar expression patterns for each of the timepoints (see the electronic supplementary material). At 30 min, two clusters of upregulated genes containing 26 and 53 genes and two clusters of downregulated genes containing 32 and 62 genes were identified. At 1 h, three clusters of upregulated genes containing 34, 41 and 21 genes and one cluster of 57 downregulated genes were found. As an example, the fold changes in one cluster of upregulated and one cluster of downregulated genes are presented in figure 1.

Functional analysis with DAVID was conducted on 80 genes found to be upregulated and 68 genes found to be downregulated at either the 30 min or 1 h timepoints. Twenty-two functional groups were found to be upregulated. A typical functional group is shown in table 1 with GO terms, *p*-value, fold enrichment and gene members. Upregulated GO categories included differentiation and developmental processes, transcriptional regulation, intracellular signalling, tyrosine phosphorylation, post-translational modification, leucine zipper transcription, apoptosis, cytoskeleton, morphogenesis, differentiation and the plasma membrane. Among the downregulated categories, there was some overlap with upregulated categories, including differentiation, cytoskeleton, kinase activity and the plasma membrane. Others were unique, including keratin, zinc and metal ion binding, metabolic activity, intracellular organelles, protein localization and angiogenesis.

Figure 1. Example of the results of the profile clustering analysis. One upregulated cluster and one downregulated cluster at 30 min are shown. Upregulation is indicated in red and downregulation in green.

Table 1. An example DAVID functional cluster (leucin-zipper transcriptional regulation). Included are the GO terms associated with this cluster, *p*-values, fold enrichment and gene members for each GO term.

functional group 7			
GO term	<i>p</i> -value	fold enrichment	gene members
IPR004827:Basic-leucine zipper (bZIP) transcription factor	0.0079	22.0	Mafk, Jun, Atf3,
SM00338:BRLZ domain:Leucine-zipper	0.0173	14.4	Mafk, Jun, Atf3,
DNA-binding region:Basic motif	0.0454	8.6	Mafk, Jun, Atf3,
DNA-binding	0.0707	6.7	Mafk, Jun, Atf3,
	0.1368	1.8	Hoxc4, Tbx2, Mafk, Zfpm2, Kin, Trim24, Jun, Atf3,
GO:0043565~sequence-specific DNA binding	0.1670	2.8	Hoxc4, Mafk, Jun, Atf3,
GO:0046983~protein dimerization activity	0.1970	3.6	Mafk, Jun, Atf3,
GO:0003700~transcription factor activity	0.2754	1.8	Hoxc4, Tbx2, Mafk, Jun, Atf3,

From the list of known genes upregulated by dynamic fluid flow at 30 min post-flow, nine were selected for validation of expression level via quantitative real-time RT-PCR. They were selected due to their importance in osteoblast function or known shear stress-responsive signalling pathways. RT-PCR data were obtained for these genes at both 30 min and 1 h post-flow and values are reported as fold change, normalized to 18S rRNA levels (figure 2 and table 2). In this set, upregulation by microarray at the 30 min timepoint was confirmed RT-PCR (more than twofold) in all cases. In addition, six of these nine genes remained upregulated by PCR at 1 h as well. However, among these, only four of six were found to be correspondingly upregulated on microarray. False positives such as these are a common concern with the large number of assessments conducted with high-throughput analysis. It demonstrates that identification of single candidate genes from high-throughput analyses needs to be confirmed with focused assays. It also indicates the advantage of systems bioinformatics approaches (as we have conducted here), which are less susceptible to false positives.

4. Discussion

While we largely focus our discussion here on those genes whose expression levels were assayed with PCR, clustering and functional grouping of microarray data may be useful for analysis of molecular pathways involved in mechanotransduction. Consistent with previous studies (Saunders *et al.* 2001; Donahue *et al.* 2003b), which showed increased prostaglandin E₂ (PGE₂) release in MC3T3-E1 cells shortly after exposure to dynamic fluid flow, our results demonstrate a 9.6-fold increase in the mRNA level of the enzyme catalysing PGE₂ production, cyclooxygenase 2 (prostaglandin-endoperoxide synthase 2; Ptg2), at 30 min post-flow. At 1 h post-flow there was a 3.1-fold increase in Ptg2. In addition, at the 30 min timepoint, there was a 3.8-fold increase in the

Figure 2. Dynamic fluid flow versus no-flow fold-change values at 30 min (filled bars) and 1 h (open bars) post-flow obtained via quantitative RT-PCR, normalized to 18S rRNA levels. PCR validation was conducted on the basis of the 30 min data.

Table 2. Dynamic fluid flow versus no-flow fold change for nine genes selected for validation via RT-PCR. Fold-change values are shown from quantitative RT-PCR, normalized to 18S rRNA levels, as well as relative array fold changes for comparison. The asterisk denotes a gene that did not show an upregulation in three or more replicate arrays.

gene symbol	gene name	30 min PCR	30 min array	1 h PCR	1 h array
Hmox1	heme oxygenase (decycling) 1	33.2	86.8	15.2	7.3
Fos11	Fos-like antigen 1	13.3	6.8	3.6	*
Ptgs2	prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2)	9.6	4.3	3.1	*
Atf3	activating transcription factor 3	7.7	13.5	5.8	5.8
Ptger4	prostaglandin E receptor 4 (subtype EP4)	3.8	4.4	1.3	*
Ier3	immediate early response 3	3.0	4.8	2.5	2.6
Slc20a1	solute carrier family 20, member 1	3.0	2.8	1.8	*
Mt2	metallothionein 2	3.0	6.5	3.4	3.8
Junb	Jun-B oncogene	2.6	4.5	1.6	*

level of PGE₂ receptor EP4 (Ptger4) mRNA. EP4 has been shown to be the PGE₂ receptor subtype critical for both the *in vivo* and *in vitro* osteogenic effects of PGE₂ stimulation upon osteoblasts (Yoshida *et al.* 2002).

Heme oxygenase 1 (Hmox1), the inducible isoform of an intracellular enzyme that catalyses the production of the signalling molecule CO, was upregulated by 33.2-fold at 30 min post-flow and by 15.2-fold at 1 h. Hmox1 induction has been

shown *in vivo* in rat ulnae subjected to short-duration compressive axial loading at a level sufficient to generate an osteogenic response (Rawlinson *et al.* 1998). Furthermore, Hmox1 upregulation has been shown in endothelial cells exposed to both steady and oscillating fluid flows (De Keulenaer *et al.* 1998). Recent data also suggest that Hmox1 induction in osteoclasts is mediated by mitogen activated protein kinase pathways and inhibits osteoclastogenesis and, in turn, bone resorption (Zwerina *et al.* 2005). Our results suggest that Hmox1 induction by flow may be an integral component of the anabolic response of MC3T3-E1 cells to cyclic mechanical loading.

Solute carrier family 20, member 1 (Slc20a1), a sodium-dependent phosphate symporter in osteoblasts important in matrix mineralization, was upregulated threefold with flow at 30 min post-flow. Nielsen *et al.* (2001) have reported increased levels of Slc20a1 mRNA during osteoblastic differentiation of MC3T3 cells, and other investigators have shown increased Slc20a1 is a marker of metabolically active osteoblasts (Suzuki *et al.* 2001; Yoshiko *et al.* 2003).

Metallothionein 2 (Mt2) was upregulated by threefold at 30 min and remained upregulated by 3.4-fold at the 1 h timepoint. Metallothioneins are involved in nitric oxide-mediated signalling by regulating intracellular zinc ion homeostasis in response to nitric oxide binding and are also cytoprotective in the setting of oxidative stress (Pearce *et al.* 2000). They may also be involved in nitric oxide-mediated osteogenic pathways in osteoblasts (Bloomfield 2001). Nitric oxide production is known to increase in MC3T3 osteoblasts in response to fluid shear stress (Bacabac *et al.* 2004), and metallothionein upregulation has been shown in dexamethasone-stimulated osteogenesis in MC3T3 cells (Miyahara *et al.* 1991).

Among transcription factors, members of the activator protein 1 (AP-1) family were found to be regulated by flow. Three AP-1 transcription factor components were also upregulated at 30 min post-flow (Fos11, Atf3, Junb), but only two remained upregulated by more than twofold at 1 h (Fos11, Atf3). The AP-1 transcription factor complex regulates various aspects of osteoblast differentiation (Wagner 2002), and the upregulation of these specific AP-1 components suggests their involvement in mediating the osteogenic response to dynamic fluid flow. Further, our data suggest that perhaps the early response to mechanical stimulation is characterized by activation of a number of signalling cascades that become progressively more refined with time post-stimulation. However, a more refined time-course experiment with more immediate RNA isolations would be necessary to confirm this observation.

In addition to AP-1 family transcription factors, immediate early response 3 (Ier3), a transcription factor regulating cell apoptosis, was upregulated by approximately threefold both at 30 min and 1 h post-flow. Ier3 upregulation in response to biaxial cyclic strain has previously been shown in aortic smooth muscle cells in a nuclear factor- κ B-dependent manner (De Keulenaer *et al.* 2002), as well as in cardiac myocytes (Schulze *et al.* 2003). This suggests that the expression of Ier3 is biomechanically controlled in a pathway that could also be activated by mechanical stimulation.

In conclusion, we have identified several novel genes involved in the early response of MC3T3-E1 cells to the anabolic stimulus of OFF. Many of these genes have been shown in prior studies to be involved in osteogenic pathways in osteoblasts or implicated in fluid shear-stress responses in various cell types. Further studies will elucidate the exact role of these genes in

dynamic-fluid-flow-regulated mechanotransduction pathways, and microarray analysis with functional grouping of genes will be critical in formulating a complete understanding of complementary intracellular processes.

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