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Oscillatory fluid flow induces the osteogenic lineage commitment of mesenchymal stem cells: The effect of shear stress magnitude, frequency, and duration

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ABSTRACT

A potent regulator of bone anabolism is physical loading. However, it is currently unclear whether physical stimuli such as fluid shear within the marrow cavity is sufficient to directly drive the osteogenic lineage commitment of resident mesenchymal stem cells (MSC). Therefore, the objective of the study is to employ a systematic analysis of oscillatory fluid flow (OFF) parameters predicted to occur *in vivo* on early MSC osteogenic responses and late stage lineage commitment. MSCs were exposed to OFF of 1 Pa, 2 Pa and 5 Pa magnitudes at frequencies of 0.5 Hz, 1 Hz and 2 Hz for 1 h, 2 h and 4 h of stimulation. Our findings demonstrate that OFF elicits a positive osteogenic response in MSCs in a shear stress magnitude, frequency, and duration dependent manner that is gene specific. Based on the mRNA expression of osteogenic markers Cox2, Runx2 and Opn after short-term fluid flow stimulation, we identified that a regime of 2 Pa shear magnitude and 2 Hz frequency induces the most robust and reliable upregulation in osteogenic gene expression. Furthermore, long-term mechanical stimulation utilising this regime, elicits a significant increase in collagen and mineral deposition when compared to static control demonstrating that mechanical stimuli predicted within the marrow is sufficient to directly drive osteogenesis.

1. Introduction

Bone structure adapts to meet the daily mechanical demands imposed during locomotion (Robling et al., 2006; Hu et al., 2012; Kwon et al., 2010). This mechanically driven process dictates synthesis of new bone by osteoblasts derived from mesenchymal stem cells (MSCs). Due to the limited lifespan and non-proliferative nature of osteoblasts *in vivo*, MSCs play a critical role in replenishing the osteoblast population by undergoing osteogenic lineage commitment (Ren et al., 2015; Chen et al., 2016; Tewari et al., 2015). Despite this important role for MSCs in bone adaptation, it is not fully understood how loading of bone at the macro scale regulates MSC differentiation within the niche. Two mechanisms have emerged whereby loading induced-MSC lineage commitment may be driven indirectly through paracrine signalling from mature bone cells or alternatively, MSCs may sense the applied mechanical stimulus directly (Riddle and Donahue, 2009; Govey et al., 2013; Hoey et al., 2011; Schaffler et al., 2014; Brady et al., 2015). Although the response of bone cells such as osteocytes to a range of mechanical stimuli predicted within the lacunar-canalicular system (LCS) is known, how MSCs respond to the dynamic mechanical environment of the niche, or whether this mechanical stimulation is sufficient to directly drive osteogenic lineage commitment remains poorly understood.

During daily ambulation, the skeletal system including the marrow experiences complex mechanical stimuli such as pressure and fluid flow-induced shear (Ren et al., 2015). Bone marrow experiences fluctuations in intramedullary pressure of up to 30 kPa, which is dependent on loading, blood flow and muscle contraction that is oscillatory in nature (Gurkan and Akkus, 2008). The pressure gradients that develop within the marrow cavity with loading force the marrow to flow imparting a shear stress to resident cells, including MSCs. Until recently, the MSC micromechanical environment was poorly understood, therefore MSCs were subjected to shear stresses known to occur within the LCS of bone such as 0.1–3 Pa (Delaine-smith et al., 2012; McCoy and O'Brien, 2010;

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Weinbaum et al., 1994; Verbruggen et al., 2014; Espinha et al., 2014). However, recent work using fluid-structure interaction models of reconstructed trabecular bone and bone marrow has demonstrated that the shear stresses imparted to MSCs are dependent on the deformation rate, bone porosity and bone marrow properties and can result in shear stresses exceeding 5 Pa (Metzger et al., 2015; Coughlin and Niebur, 2012). This therefore indicates that *in vitro* experimental studies to date have utilised shear magnitudes that represent the lower spectrum of the range that MSCs can experience *in vivo* with loading.

Direct mechanical stimulation of MSCs *in vitro* can play a positive role in directing cell lineage commitment. In particular, a number of studies have explored the effect of fluid shear on osteogenic gene expression employing variable biophysical regimes. Cyclooxygenase-2 (Cox2), a necessary enzyme involved in bone anabolism, is upregulated in as little as 30 min following either steady, pulsatile or oscillatory fluid flow stimulation at shear stress magnitudes of 0.1–1 Pa (Becquart et al., 2016; Zhang et al., 2002; Glossop and Cartmell, 2009). Similarly, fluid flow-induced upregulation of the osteoblastic master transcription factor, Runx2, occurs in response to both steady and oscillatory flow; however, this was in the presence of osteogenic supplements (Hoey et al., 2012; Kim et al., 2014; Li et al., 2004; Grellier et al., 2009). MSCs exposed to steady flow of less than 0.3 Pa magnitude demonstrated enhanced expression of the bone matrix proteins such as Opn, Ocln, BSP and Col1α1 when cultured for up to 21 days (Grellier et al., 2009). However, employing a pulsatile or oscillatory flow results in an upregulation of the gene expression of these bone matrix proteins within an hour of stimulation (Sharp et al., 2009, McGarry, 2005). Furthermore, due to dynamic nature of locomotion, OFF is considered more physiologically mimetic compared to steady and pulsatile regimes, which have predominately been used in bioreactor based tissue engineering strategies (Jacobs et al., 1998). Loading-induced bone formation *in vivo* has a magnitude-frequency interdependence such as the coupling of high magnitude-low frequency stimulation and vice versa (Turner et al., 1994; Nagaraja and Jo, 2014). Interestingly, most OFF studies utilize frequency of 1 Hz in order to mimic the ambulatory pace, however the effect of a range frequencies related to human motion has not been investigated (Danion et al., 2003; Arnsdorf et al., 2010; Riddle et al., 2008; Riddle et al., 2007; Riddle et al., 2006). Given the variable MSC response to fluid flow, a systematic study that investigates the effect of the magnitude, frequency and duration of physiologically relevant OFF on osteogenic responses is required.

It is currently unclear whether fluid flow induced shear predicted to occur within the marrow during daily ambulation is sufficient to directly drive osteogenic lineage commitment of MSCs. Therefore, the objective of this study is to conduct a systematic analysis of oscillatory fluid shear stress magnitude, frequency and duration on early osteogenic responses and to determine whether these mechanical stimuli are sufficient to drive osteogenic lineage commitment of mesenchymal stem cells in the long term. Elucidating how marrow mechanical environment affects osteogenic responses in MSCs will enable the development of advanced strategies for orthopaedic bioreactor based tissue engineering and will provide a platform to delineate the mechanisms of loading-induced MSC osteogenic differentiation and bone formation.

2. Materials and methods

2.1. Cell culture

All materials were purchased from Sigma unless otherwise stated. A murine mesenchymal stem cell line (C3H10T1/2) was cultured on fibronectin (10 μg/ml) coated glass slides in low glucose DMEM supplemented with 10% foetal bovine serum (FBS: Biosera) and 1% penicillin-streptomycin (P/S) unless otherwise stated. For short term fluid flow stimulation, cells were cultured for 24 h under standard conditions followed by 48 h of serum starvation (0.5% FBS) supplemented with 0.3 nM dexamethasone, 0.025 mM L-ascorbic acid and 10 mM β-glycerolphosphate. These concentrations represent minimal levels for the support of osteogenesis, thereby allowing greater scope to investigate the effect of a biophysical versus a biochemical stimulus. Regarding long-term fluid flow stimulation, the cells were cultured in similar conditions, except supplemented with 2% FBS and 2% P/S. To demonstrate that the these minimal osteogenic supplements do not induce osteogenic differentiation of MSCs alone, for long term mechanical stimulation, an additional 'Control' group consisting of standard growth media with no additional osteogenic supplements was included.

2.2. Fluid flow mechanical stimulation

OFF was achieved by applying an oscillatory pressure driven flow via a syringe pump (Alladin 1660) to the custom-made PPFC (Fig. 2A). The volumetric rate of flow (Q) necessary for a given shear stress was calculated using equation (Eq. (1)) and validated and adjusted for any pump losses based on the flow output (see Supplemental methods). To systematically delineate the effect of fluid shear stress magnitude, frequency and duration on early osteogenic responses of MSCs, a series of fluid flow regimes were employed and are presented in Table 1. These enabled comparisons of flow regimes with a constant number of loading cycles i.e. 0.5 Hz, 4 h against 1 Hz, 2 h and 2 Hz, 1 h. These flow regimes were modelled on a previous systematic investigation of fluid flow on osteocytes (Li et al., 2012). No flow control samples, i.e. not subjected to fluid shear for both short and long-term stimulation were assembled in the chambers to reproduce handling effects for a matched duration of mechanical stimulation (no flow control). To determine whether oscillatory fluid shear could induce osteogenic lineage commitment, and based on our results from the short-term systematic analysis, MSCs were subjected to two separate flow regimes (FR) over a long-term duration as described in Fig. 1. FR1 consisted of a fluid shear of magnitude 1 Pa and frequency 1 Hz (most common flow regime for bone cells) while FR2 consisted of a fluid shear of magnitude 2 Pa and frequency 2 Hz. For both long-term FRs, MSCs were exposed to fluid shear on Days 1, 2, 4, and 5 for 4 h a day followed by an additional 14 days of static culture.

2.3. Quantitative real-time PCR

Immediately post-flow, cells were lysed using TRI Reagent® (Sigma Aldrich) and mRNA was isolated according to the manufacturer's protocol. One microgram of RNA was reverse transcribed into cDNA using High Capacity cDNA kit (Life Technologies). Quantitative polymerase chain reaction (qPCR) was performed using SYBR Select Mastermix with ROX passive dye (ThermoFisher 4472903). The expression of 18S, Cox2, Runx2 and osteopontin (Opn) was quantified using primers detailed in Table 2 (Sigma). The amplification was performed with an ABI7500 Fast Real Time PCR machine. Each sample was normalised to reference gene 18S and static control using relative quantification method.

2.4. Osteogenic assays

Cells were fixed in formalin for 10 min. For collagen staining, cells were incubated in 0.1% PicroSirius Red solution for 1 h at room temperature. After washing twice in 0.5% acetic acid and water, samples were mounted with DPX mounting medium. Calcium staining was performed using 2% Alizarin Red solution. The bound dye for both calcium and collagen was observed under light microscopy. The bound Alizarin Red was used to quantify the calcium content by extraction using 10%v/v acetic acid and measuring the absorbance at 405 nm.

3. Results

3.1. Parallel plate fluid flow bioreactor design and validation

The chamber design was optimised to minimise pressure changes and the entrance length while achieving a homogenous fluid flow-induced shear stress. This was achieved by varying the height of the channel to determine the theoretical changes in entrance length (L_e), pressure drop across the chamber (ΔP) for a

Table 1
Experimental conditions for short-term fluid flow stimulation.

Shear stress	Frequency		
	0.5 Hz	1 Hz	2 Hz
1 Pa (44.9 ml/min)	2 and 4 h	2 h	1 and 2 h
2 Pa (89.8 ml/min)	2 and 4 h	2 h	1 and 2 h
5 Pa (224.5 ml/min)	2 and 4 h	2 h	1 and 2 h

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