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Focal adhesion kinase signaling regulates anti-inflammatory function of bone marrow mesenchymal stromal cells induced by biomechanical force

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Abstract

Mesenchymal stromal cells (MSCs) have tremendous potential for use in regenerative medicine due to their multipotency and immune cell regulatory functions. Biomimetic physical forces have been shown to direct differentiation and maturation of MSCs in tissue engineering applications; however, the effect of force on immunomodulatory activity of MSCs has been largely overlooked. Here we show in human bone marrow-derived MSCs that wall shear stress (WSS) equivalent to the fluid frictional force present in the adult arterial vasculature significantly enhances expression of four genes that mediate MSC immune regulatory function, *PTGS2*, *HMOX1*, *IL1RN*, and *TNFAIP6*. Several mechanotransduction pathways are stimulated by WSS, including calcium ion (Ca^{2+}) flux and activation of Akt, MAPK, and focal adhesion kinase (FAK). Inhibition of PI3K-Akt by LY294002 or Ca^{2+} signaling with chelators, ion channel inhibitors, or Ca^{2+} free culture conditions failed to attenuate WSS-induced COX2 expression. In contrast, the FAK inhibitor PF-562271 blocked COX2 induction, implicating focal adhesions as critical sensory components upstream of this key immunomodulatory factor. In co-culture assays, WSS preconditioning stimulates MSC anti-inflammatory activity to more potently suppress TNF- α production by activated immune cells, and this improved potency depended upon the ability of FAK to stimulate COX2 induction. Taken together, our data demonstrate that biomechanical force potentiates the reparative and regenerative properties of MSCs through a FAK signaling cascade and highlights the potential for innovative force-based approaches for enhancement in MSC therapeutic efficacy.

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Contributors

H.J.L. and P.L.W. designed the study and methodology. H.J.L., M.F.D., and A.E. completed the experiments and analyzed the data. S.D.O. and C.S.C. provided guidance on experimental design and direction. H.J.L. and P.L.W. wrote the manuscript and made final corrections prior to submission. All authors read and approved the final article.

Disclosure of potential conflicts of interest

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Keywords

Anti-inflammatory; COX2; FAK; Immunomodulation; Mesenchymal stromal cells; Shear stress

1. Introduction

Mesenchymal stromal cells (MSCs) are multipotent osteoprogenitor cells capable of differentiation into a variety of cell types, including adipocytes, osteoblasts, and chondrocytes [1]. In addition to their differentiation potential, MSCs have been reported to regulate the immune response in many diseases through the production of various paracrine factors [2–5]. MSCs secrete a broad spectrum of soluble factors that can alter the local milieu by contributing to angiogenesis, tissue repair, cytoprotection, native cell growth, and inflammatory suppression [6,7]. Chemokines, anti-inflammatory cytokines, growth factors and other bioactive molecules secreted by MSCs regulate function and behavior of macrophages and other immune cells [7,8]. Due to the reparative and regenerative properties of MSCs, therapeutic application of MSCs is being tested in a number of clinical trials for various indications including cardiovascular diseases [9,10], neurodegenerative diseases [11], transplantation-related rejection or complication [12–14], autoimmune diseases [15] and metabolic disorders [16,17]. However, the important therapeutic features of self-renewal, multipotentiality, and immune modulation of MSCs become limited when these cells are introduced into in vitro culture, and MSCs progressively senesce [18,19].

Under physiological conditions, all adherent cells are surrounded by an extracellular matrix (ECM), which provides support, trophic signaling and biophysical cues, eventually defining fundamental cell properties, including cell cycling, survival, paracrine activity, motility, homing behavior, and cell fate [20,21]. These native, inherently mechanical, environments direct stem cell differentiation and function [22]. For example, cell culture substrates and scaffolds with bio-inspired topographic features drive MSCs into spatial arrangements that regulate their morphology, stemness, and fate [23–25]. In a recent study, we demonstrated that bone marrow-derived MSCs exposed to wall shear stress (WSS) mimicking vascular biomechanical forces up-regulated anti-inflammatory mediators, including COX2, HO-1, and prostaglandin E₂ (PGE₂). Furthermore, MSCs treated ex vivo with shear stress exhibited enhanced neuroprotective and anti-inflammatory effects in the injured rat brain following traumatic brain injury [26]. These findings promise to provide new methods to improve potency of MSCs used in cellular therapy, yet the molecular mechanism that drives the activation of anti-inflammatory signaling downstream of shear stress is incompletely understood.

Using primary human bone marrow MSCs, we investigate the mechanisms that regulate key mediators of MSC anti-inflammatory function. We demonstrate that Ca²⁺, Akt, MAPK, and focal adhesion kinase (FAK) signaling rapidly respond to WSS and that FAK is a critical regulator of flow-induced COX2 protein expression. Importantly, FAK-COX2 signaling is required for MSC immunomodulatory function, as inhibition of FAK abrogates COX2 induction and the ability of MSCs to suppress inflammatory cytokine production by activated immune cells.

2. Materials and methods

2.1. Cell culture and pharmacological reagents

Bone marrow MSCs were derived from whole bone marrow from independent human donor (AllCells). Cells were isolated and maintained as described previously [26]. Briefly, enriched mononuclear cells by phase separation in Ficoll-Paque were resuspended for immediate expansion in complete culture medium consisting of MEM- α (Thermo Scientific), 20% fetal bovine serum (Atlanta Biologicals), 100 units/ml penicillin (Gibco), 100 μ g/ml streptomycin (Gibco), and 2 mM L-glutamine (Gibco). Nonadherent cells were removed after 2 days. Adherent colonies were expanded further and frozen as Passage 1. Expression of cell surface markers defined by the International Society for Stem Cell Therapy, including CD90 (+), CD73 (+), CD45 (–), CD34 (–), HLADR (–), CD19 (–), and CD11b (–) was confirmed by flow cytometry [26,27]. Thawed MSCs were plated at 1×10^5 cells/ml, and medium was changed every three days. At 80% confluence, cells were passaged into IBIDI channels (μ -slide VI 0.4) at a density of 3×10^6 cells/ml for biochemical analysis. Following attachment to the culture surface, media flow was applied to produce laminar shear stress of 15 dyn/cm², as detailed in the Microfluidics section below. Complete MEM- α culture medium was used, with the exception of experiments requiring Ca²⁺ free media prepared as above with a different base medium (MEM media, no calcium, no glutamine; Thermo Scientific).

All compounds targeted to MSCs were added 30 min to 1 h prior to application of WSS, were present for the duration of WSS exposure, and were washed out with fresh media prior to further experimental procedures unless noted otherwise. BAPTA-AM (Tocris) was applied to cells at a concentration of 10 μ M as an intracellular calcium chelator. EGTA (ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, Acros Organics) was used at a concentration of 5 mM as an extracellular calcium chelator. Gd³⁺ (Gadolinium chloride, Tocris) was used at 30 μ M to block ion channels. LY294002 (Cayman Chemical) was applied to cells at a concentration of 10 μ M to inhibit PI3K. PF-562271 (Selleckchem) was used at 10 μ M to block FAK. Specific inhibitors of EP₂ (PF-04418948, Cayman Chemical) and EP₄ (L-161,982, Cayman Chemical) were applied at 10 μ M during MSC co-culture with splenocytes. The stabilized analog of PGE₂ (dimethyl-PGE₂, Cayman Chemical), EP₂ selective agonist butaprost (Cayman Chemical), or the EP₄ agonist TCS 2510 (Fisher Scientific) were added to splenocyte cultures at 10 μ M.

2.2. Microfluidics

Microfluidics devices (6 channel μ -slide VI^{0.4}, IBIDI LLC) were used in all experiments. Prior to seeding cells in the device, the channels were coated for 30 min using 100 μ g/ml fibronectin (Invitrogen Life Sciences) at 37 °C. Human bone marrow MSCs were then seeded and allowed to attach for 18 h. Following attachment, shear stress was applied using a 12-roller peristaltic pump (REGLO analog MS4/12, Ismatec) at 15 dyn/cm² for up to 6 h, as previously described [26]. Static controls were plated in microfluidic slides under no-flow conditions, with the exception of fluid movement associated with manual medium change.

2.3. RNA extraction and quantitative RT PCR

Total RNA was extracted from 90,000 cells with the RNeasy Micro Kit (Qiagen). Reverse transcription of RNA was performed using Applied Biosystems Multiscribe DNA polymerase, and Real-time Taqman PCR (Applied Biosystems) was performed in 10 μ l reactions with primers provided by Applied Biosystems. For calculation of fold change, cycle thresholds (Ct) were determined using SDS 2.2.1 software (Applied Biosystems), and mRNA expression was normalized to GAPDH transcript and the control sample.

2.4. Calcium imaging

Cells were plated into IBIDI slides at a density of 10^6 cells/ml. After attachment, cells were washed with isotonic Tyrode's solution (139 mM NaCl, 3 mM KCl, 17 mM NaHCO₃, 12mM D-glucose, 3 mM CaCl₂, and 1 mM MgCl₂) and incubated with the fluorescent calcium indicator 5 μ M Fluo-4 AM (F14201; Invitrogen) in isotonic Tyrode's solution for 5 min at 37°C. Fresh phenol red free MEM- α medium was applied, and cells were placed in an environmental chamber maintained at 37 °C, 5% CO₂ for imaging. Successive images were collected at a 385-msec time interval for 77 s (201 images total) using MetaMorph v7.7.9.0 on an Olympus IX81 fluorescent microscope equipped with an Andor iXon X3 885 EMCCD camera. Shutter was left open for the duration of imaging for rapid acquisition of frames, with the tradeoff that continuous excitation accelerated photobleaching of the Fluo-4 AM signal [28]. WSS was applied 5 s after initiation of image acquisition and thus represents static culture at start time. Images were subsequently analyzed for integrated intensity using manual selection of individual cell boundaries (a region of interest, ROI) and calculated for the average fluorescence intensity / ROI (F) with arbitrary units (AU) using MetaMorph software. To account for variations in baseline F (AU) among cells on the same slide and greater variation in F across cells from independent experiments on different days, F at each time point was normalized to its initial (the first digital frame acquired for each experiment) fluorescence intensity (F₀): $(F - F_0) / F_0$, as described in a previous study [29]. The normalized fluorescence response to WSS was characterized by quantifying the percentage of responding cells. Responding cells were defined as cells with a positive value after normalization. The percentage of cells per field of view was plotted during 77 s WSS. Videos were compiled for visualization purposes using Amira 6.1.1 software (LEI) using a bilateral filter and block face correction to smoothen and reduce slice-based intensity fluctuation. Static and WSS images were treated identically, with the exception that the Static intensity was increased post-acquisition to match the starting intensity of the WSS movie. A custom look up table was applied to colorize the videos.

2.5. Immunoblotting

Cells were harvested in RIPA cell lysis buffer (GenDepot, R4100-010) with protease (Thermo Scientific, 88665) and phosphatase inhibitor cocktail (Sigma, P5726). After protein determination by protein assay dye (BioRad, 5000006), normalized lysate sample amounts were mixed with Laemmli's SDS sample buffer (GenDepot, L1100-001) and separated by SDS/PAGE on poured 10% bis-acrylamide (BioRad, 161-0156) mini gels (Novex Cassette, 1.0 mm, 10 well, Thermo Fisher, NC2010) with 4% stacking. Gels were then wet transferred onto a nitrocellulose membrane (0.45 μ m, BioRad, 162-0115) and analyzed by Western

blotting using West Pico chemiluminescent substrate (Thermo Scientific, 34080) or Li-cor WesternSure Premium substrate (Fisher, 50-489-552). Immunoblotting was prepared by standard procedures using rabbit anti-COX2 (Abcam, ab15191), rabbit anti-TSG6 (Abcam, ab128266), rabbit anti-IL1Ra (Abcam, ab124962), rabbit anti-heme oxygenase 1 (Abcam, ab13243), rabbit anti-phospho-Akt (Ser 473, Cell Signaling, 9271), rabbit anti-Akt (Cell Signaling, 9272), rabbit anti-phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204, Cell Signaling, 9101), rabbit anti-p42/44 MAPK (Cell Signaling, 9102), rabbit anti-FAK (Cell Signaling, 3285), rabbit anti-pFAK (Tyr397, Cell Signaling, 3283), and mouse anti- β -actin (Santa Cruz, sc-47778) antibodies. Gel images were scanned and the density of the protein bands was quantified as a ratio to the total protein or actin loading control by MCID Analysis 7.1 software (InterFocus Imaging Ltd.) for films or by Image Studio software for the Li-cor C-DiGit chemiluminescent blot scanner.

2.6. Immunofluorescence microscopy

MSCs were fixed on microfluidics channels in 4% paraformaldehyde for 15 min, washed with PBS, and stored overnight at 4 °C. Slides were permitted to come to room temperature the next morning, sticky channel overlays removed by single edge razor blade, and cells permeabilized in PBS with 0.2% Triton X-100 for 5 min at room temperature. Cells were incubated with Image-iT FX signal enhancer (Thermo Fisher, I36933) for 30 min at room temperature. Rabbit anti-pFAK polyclonal antibody (1:100 dilution, anti-pFAK Y397, Cell Signaling 3283S) was applied for labeling activated FAK for 1 h at room temperature in 2.5% BSA-0.1% Triton X-PBS. Slides were then washed with PBS. Cells were incubated with a 1:1000 dilution of goat anti-rabbit IgG Alexa Fluor 488 secondary antibody (Invitrogen, Cat. No. A11008) for 1 h and washed in PBS. Cells were subsequently incubated with 25 μ M DRAQ5 for 30 min at room temperature. Coverslips were mounted with Prolong Gold (Invitrogen). Images were captured by a Leica TCS SP5 confocal microscope with a Leica 63 $\times$ oil objective lens (NA 1.4) and analyzed with LAS Advanced Fluorescence software (Leica) and ImageJ 1.50i (NIH) to measure fluorescence intensity of the green channel.

2.7. TNF- α suppression assay

Human bone marrow MSCs were cultured at a density of 2×10^5 cells/ml. Murine splenocytes were isolated from 2 to 4 month old C57BL/6 J mice for co-culture at 6×10^6 cells/ml with MSCs immediately after shear stress preconditioning. Briefly, spleen was mechanically dissociated by pushing through a 70- μ m strainer, lysed in Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich), quenched with 2% BSA in PBS and pushed through a 40- μ m strainer, centrifuged to pellet and resuspended after PBS washes in medium for culture with MSCs. Splenocyte-MSC co-cultures were plated at a ratio of 30 to 1. Lipopolysaccharide (LPS; Sigma Aldrich) was applied at 1 μ g/ml 30 min after plating. Following 18 h incubation, supernatant was collected and analyzed for TNF- α using the Mouse TNF- α Quantikine ELISA kit (R & D Systems) following standard R & D Systems protocol.

2.8. Statistical analyses

Independent experiments were conducted on different days with primary human bone marrow cell lines. All data were analyzed with SigmaPlot 12.5 for statistical significance and are reported as mean $\pm$ SEM. Parametric tests were used when data met assumptions of homoscedasticity and normality; otherwise, nonparametric tests were employed.

3. Results

3.1. Shear stress regulates anti-inflammatory factors independently of Ca^{2+} signaling

We recently reported that human bone marrow-derived MSCs respond to laminar shear stress by increased immune modulatory activity [26]. When used as a transient conditioning method *ex vivo*, shear stress enhanced potency of MSCs administered to rats to control inflammatory response in the brain following traumatic brain injury. To investigate the mechanotransduction signaling pathways responsible for the observed change in immunomodulatory function, MSCs were cultured in microfluidics capable of producing uniform laminar flow at a WSS of 15 dyn/cm² typical of human arterial stresses. Consistent with previous observations, key mediators of MSC anti-inflammatory function, *PTGS2*, *HMOX1*, *IL1RN*, and *TNFAIP6* genes, were significantly upregulated in MSCs exposed to WSS for 3 h and 6 h (Fig. 1A; $n = 4$; Kruskal-Wallis One Way ANOVA, $P < 0.001$). Among many signal transduction pathways induced by WSS, Ca^{2+} has been thought to play a role as a rapid responder to shear stress [29,30]. Thus, we measured Ca^{2+} signaling induced by WSS using a cell permeant dye that increases in fluorescence upon binding to Ca^{2+} , Fluo-4 AM. Fluo-4 AM-loaded MSCs were monitored by time lapse imaging for changes in fluorescence during a period of 77 s of static culture or 5 s of static culture followed by a 77 s exposure to WSS with 15 dyn/cm². Although Ca^{2+} flashes were observed within static conditions, exposure to WSS induced more intense signaling across a greater number of cells (Fig. 1B, C and Supplementary Video 1). Normalized fluorescence traces for each cell in the field of view (~ 30 cells) demonstrated the occurrence of transient fluorescent increases upon the onset of WSS. Elevated Ca^{2+} levels were sustained throughout the WSS exposure and could be truncated by several chelators or inhibitors of Ca^{2+} flux, including the cell permeant chelator BAPTA-AM, the extracellular Ca^{2+} chelator EGTA, and the ion channel inhibitor Gd³⁺ (Supplementary Fig. 1A). In hematopoietic stem and progenitor cells of the embryo, we found that WSS acted through a Ca^{2+} mediated pathway to increase production of PGE₂, a metabolic product of arachidonic acid metabolism [31]. We have shown previously that therapeutic benefit of MSCs correlates with the secretion of PGE₂ and is stimulated by WSS [26,32]. COX2, encoded by the *PTGS2* gene, is the rate-limiting enzyme in PGE₂ synthesis; thus, we tested the dependence of COX2 expression on cytosolic Ca^{2+} by sequestration with BAPTA-AM, the compound that was most effective at reducing cytosolic Ca^{2+} increase in MSCs (Supplementary Fig. 1B; Friedman Repeated Measures with Tukey multiple comparisons, $P < 0.05$). WSS increased COX2 protein level 6 h after initial exposure (Fig. 1D, E; $n = 3$, Two Way ANOVA with Holm-Sidak multiple comparisons, $P < 0.01$). Unlike embryonic tissues containing mixed cell types studied previously, WSS-dependent expression of COX2 was not significantly reduced by blocking Ca^{2+} with BAPTA-AM. This suggested that MSCs rely on Ca^{2+} -independent mechanisms for COX2 upregulation in response to WSS.

3.2. Akt is activated by flow but does not dictate COX2 expression

ERK and Akt are two well-known shear-responsive signaling molecules, and both kinases are quickly activated by WSS in endothelial cells [33,34]. ERK and Akt have been shown to be sensitive to mechanosensors such as caveolae, cadherins, cell-cell adhesion molecules, and Ca^{2+} signaling [33,35–38]. Fluid shear stress elevated *PTGS2* expression in osteoblasts through activation of phosphatidylinositol 3-kinase (PI3K)-Akt [39], and was recently shown to induce COX2 expression and prostacyclin release from endothelial cells via a platelet endothelial cell adhesion molecule (PECAM-1)-PI3K-dependent pathway [40]. Thus, we hypothesized that PI3K/Akt may contribute to regulation of COX2 in MSCs. We first evaluated activation of ERK and Akt by WSS. WSS resulted in profound activating phosphorylation of Akt and ERK within 5 min to 1 h of WSS (Fig. 2A, B; $n = 3$, One Way ANOVA with Holm-Sidak multiple comparisons, $P < 0.05$). In contrast to a prior report in osteoblasts [39], phosphorylation of Akt was not significantly attenuated by BAPTA-AM treatment or culture in Ca^{2+} -free medium, suggesting that Ca^{2+} signaling is not the chief regulator of Akt and ERK activation by WSS in MSCs (Supplementary Fig. 2). To investigate whether Akt contributes to increased COX2 expression, the PI3K inhibitor LY294002 was applied for 30 min before and throughout the duration of WSS. As shown in Fig. 2C and D, LY294002 did not significantly reduce the expression of COX2. We conclude that PI3K/Akt does not play a major role in WSS-mediated upregulation of COX2 in MSCs.

3.3. Activation of FAK promotes induction of COX2

Focal adhesions serve as organizing centers for signaling machinery that transduces information from the outside of the cell into chemical and genetic messages that are interpreted within the cytoplasm and nucleus. The tyrosine kinase FAK is one of many constituents that localize to focal adhesions, where integrins, actin, and various other scaffolding molecules, GTPases, and enzymes such as kinases, phosphatases, proteases, and lipases gather and interact [41]. FAK has been shown in previous reports to be required for shear-induced *PTGS2* and COX2 in endothelium and osteoblasts [39,40]. We therefore hypothesized that FAK might also mediate COX2 mechanoresponse in MSCs. Autophosphorylation of FAK at Tyr 397 (pFAK-Y397) indicative of the first stages of FAK activation appeared elevated within 5 min of WSS initiation by immunofluorescence microscopy (Fig. 3A). To quantitatively assess change in FAK phosphorylation, pFAK-Y397 signal intensity was measured and the percentage of cells exhibiting clustering of pFAK were determined. WSS produced marked change in the distribution of pFAK, leading to a significant level of clustering by 6 h after initiation of flow (Fig. 3B; Kruskal Wallis ANOVA with Tukey multiple comparisons, $P < 0.05$). Overall expression of pFAK was only modestly elevated and was less dramatic than the assembly of pFAK clusters. Phosphorylated FAK-Y397 was also compared to total FAK levels in static culture and at 5 min, 30 min, and 6 h after WSS initiation by Western blotting. Measurement of pFAK-Y397 showed no increase as determined by immunoblotting of total protein in the cell population. Notwithstanding the lack of overall pFAK increase, the FAK inhibitor PF-562271 reduced pFAK-Y397 in static and WSS cultures (Fig. 3C, D; $n = 3$, Two Way ANOVA with Holm-Sidak multiple comparisons, $P < 0.001$) and blocked flow-induced upregulation of COX2 and HO-1 expression at 6 h ($n = 4$, unpaired t -test, $P < 0.05$). Thus, although pFAK-Y397 levels do not appear to be significantly increased, confocal immunofluorescence and FAK

inhibitor studies strongly suggest that FAK is a critical regulator of COX2 expression in response to flow. Cells cultured in suspension also showed reduction in phosphorylation of FAK and COX2 protein levels, further supporting the importance of focal adhesions in control of COX2 expression. Together, these data show that FAK is required for COX2 regulation by fluid flow.

3.4. Immunomodulatory activity of MSCs requires FAK signaling

FAK is known to influence actomyosin contractility, migration, cell survival, proliferation, angiogenesis, and invasive behaviors [41,42]. Our data raised the possibility that FAK might also direct immune regulatory functions of MSCs through signaling that governs COX2-PGE₂ activity. To evaluate the effects of a FAK-initiated signaling cascade on MSC immunomodulatory function, we established co-cultures of human bone marrow MSCs with murine immune cells isolated from the spleen as described previously [26]. Immune cells from the spleen consisting of macrophages, neutrophils, NK, B, and T cells were stimulated with lipopolysaccharide (LPS) and monitored for pro-inflammatory TNF- α cytokine production. TNF- α is predominantly produced by activated M1-type macrophages but other immune cells can also produce TNF- α , including CD4⁺ T cells and NK cells. Following 18 h co-culture with LPS, species-specific ELISA was used to measure TNF- α originating from the murine splenocytes. LPS-treated splenocytes were activated to produce large amounts of TNF- α (Fig. 4A). Ectopic administration of a stabilized analog of PGE₂ (dimethyl-PGE₂), the EP₂ selective agonist butaprost, or the EP₄ agonist TCS 2510 were highly effective in reducing TNF- α secretion by splenocytes (Fig. 4A; n = 3, Two Way ANOVA with Holm-Sidak multiple comparisons, P < 0.001). In parallel assays, MSCs were cultured under static conditions or were transiently exposed to WSS for 3 h, then placed in co-culture with activated splenocytes. TNF- α secretion was significantly reduced by interactions with MSCs, regardless of mechanical preconditioning (Fig. 4A; n = 6, Two Way ANOVA with Holm-Sidak multiple comparisons, P < 0.001). We found that preconditioning of MSCs with WSS for 3 h enhanced potency in TNF- α suppression beyond that of static cultured MSCs (Fig. 4A; n = 6, Mann-Whitney Rank Sum test, P < 0.05). Importantly, inactivation of FAK signaling by treatment with PF-5622771 truncated the enhanced anti-inflammatory activity of shear-preconditioned MSCs, rendering the potency similar to that of static cultured MSCs. Selective inhibitors of the PGE₂ G-protein coupled membrane receptors EP₂ (PF-04418948) and EP₄ (L-161,982) produced similar reductions in potency. Taken together, we conclude that FAK-COX2 signaling plays a critical role in MSC immunomodulatory function and propose a model of mechanotransduction that enhances immune regulatory activity of MSCs in response to shear stress (Fig. 4B).

4. Discussion

In the present study, we show that flow initiates a FAK-COX2 signaling cascade required for MSC immunomodulatory activity. This signaling is critical for the ability of MSCs to suppress pro-inflammatory cytokine production by inflammatory cells. Our study demonstrates that WSS induces influx of intracellular free calcium and activation of FAK, Akt, and MAPK in MSCs. Inhibition of Ca²⁺ transients by treatment with chelators, an ion channel blocker, or culture in Ca²⁺ free medium fails to block COX2 induction. Importantly,

compound-based inhibition of FAK reduces COX2 expression, along with concomitant decrease in TNF- α suppression potency. Targeting receptors of one of the chief mediators of MSC immune regulatory function, PGE₂, also interrupted the ability of MSCs to suppress immune cell activation. Together, these data highlight the importance of focal adhesions in the MSC response to shear stress and in MSC immunomodulatory function via COX2-PGE₂ paracrine signaling.

Shear stress is sensed and translated into biochemical signals by various mechanosensors, including adhesion molecules like integrin [43] and PECAM-1 [44], GTP-binding proteins [45], caveolae [33], glycocalyx [46], and ion channels [47]. Protein tyrosine kinases such as FAK play a central role in translating integrin signals into chemical and genetic responses and can be found co-clustered at focal adhesions [48]. We find that WSS of 15 dyn/cm² alters clustering and distribution of pFAK-Y397 in MSCs. Unlike the apparent change at the subcellular level by microscopy, immunoblotting analysis failed to detect an overall upregulation in phosphorylated FAK. FAK has been shown previously to be phosphorylated within one to several minutes after initial exposure to fluid shear stress, but it is also apparent that the subcellular localization of pFAK is critical to its function [49]. Importantly, our data show that inhibition of FAK truncates the flow-enhanced immunomodulatory activity of MSCs and blocks the induction of COX2. Complete inhibition of flow-induced COX2 was unique to the FAK inhibitor, as targeting other mechanosensitive pathways such as Ca²⁺ and PI3K/Akt failed to significantly reduce COX2 expression. One caveat to these experiments is the potential for off-target effects of PF-5622771, a risk inherent to any pharmacological approach. Yet, PF-5622771 is one of the most highly selective and potent inhibitors of FAK available, with > 10–100-fold selectivity to FAK over Pyk2 tyrosine kinase and cyclin dependent kinases (CDK1, 2, and 3), targets with little evidence in the literature for COX2 regulatory roles. Together, these data strongly suggest that signaling at focal adhesions is important for the capacity of MSCs to regulate COX2 and the immune system.

A diverse range of mitogenic and stress stimuli modulate *PTGS2* mRNA abundance through transcriptional activation and RNA decay [50]. Although sensing of extracellular Ca²⁺ modulates transcriptional induction of COX2 in osteoblasts and fibroblasts [51–53], we did not observe any significant effects of intracellular Ca²⁺ transients on COX2 abundance. Instead, we show that COX2 expression is dependent upon FAK signaling, which elevates *PTGS2* transcript and COX2 protein. *PTGS2* mRNA is quickly degraded and its stability is regulated by RNA binding proteins that recognize elements in the 3'-untranslated region (3'UTR) of the *PTGS2* transcript [50]. Src-family kinases can be activated by FAK-Y397 phosphorylation or directly by integrin clustering before and independently of FAK activation [54]. Interestingly, stabilization of *PTGS2* mRNA has been shown to rely on Src, which phosphorylates the RNA-binding protein CUGBP2 to enhance its interaction with AU-rich regions in the *PTGS2* 3'UTR [55]. Enforced expression of CUGBP2 or constitutively active c-Src leads to stabilization of COX2. FAK also is directly linked to COX2 transcriptional regulation in mechanically stressed human periodontal ligament cells, thereby promoting PGE₂ production through control of COX2 abundance [56]. The intermediate transcription factor(s) responsible for increased COX2 in MSCs has not yet been identified. In our prior report, we showed that inhibition of NF- κ B blocks shear stress-

enhancement of MSC immunomodulatory activity [26]. NF- κ B and AP-1 transcriptional complexes have well-documented roles downstream of shear stress and have been found to be regulated by FAK and Src [57–59]. Consistent with the notion that NF- κ B may contribute to flow-induced COX2 regulation by a FAK signaling cascade, FAK has been shown to be required for fluid shear stress-induced degradation of NF- κ B inhibitors I κ B α and I κ B β and subsequent NF- κ B nuclear localization in osteoblasts and endothelium [59,60]. Future studies will be required to fully elucidate the key transcriptional regulators in the FAK-COX2 pathway. It is likely, however, that NF- κ B, C/EBP, AP-1, CREB, and other transcription factors [61] work in concert to dictate the expression of a repertoire of growth factors, cytokines, and kinases that reinforce anti-inflammatory functions of MSCs [62].

We also found that WSS stimulated ERK phosphorylation, consistent with previous studies that have implicated the MAPK pathway as an important intracellular signaling component of WSS-induced mechanotransduction in human MSCs [63,64]. A body of literature shows that MAPK-ERK signaling plays roles in mediating differentiation of MSCs by growth factor-based methods and by mechanical approaches [63,65–67]. Other kinases, such as p38 MAPK and Akt, are primarily associated with immunomodulatory function and tissue repair properties of MSCs [68,69]. Indeed, decreased phosphorylation activity of p38 MAPK caused decline in performance of aged MSCs via reduced production of PGE₂ [68]. Interestingly, ectopic expression of any of the MAP kinases (ERK, JNK, or p38) can induce expression of *PTGS2* transcript in fibroblasts [70]. We have not directly examined the contribution of MAPK to enhanced production of PGE₂ in the MSC response to flow. Since MAPK lies downstream of FAK and MAPK has been directly linked to COX2 upregulation in other contexts, it is likely that MAPK and other regulators of transcription and RNA stability converge to determine COX2 abundance in response to the mechanical stress described in the current study. We speculate also that the MAPK signaling cascade could represent initiation of an early program of differentiation toward osteoblastic fate, and the long-term implications for lineage maturation of a transient WSS exposure will require more careful examination in future work.

Various therapeutic applications expose MSCs to distinct mechanical environments. Expansion, preconditioning, delivery, encapsulation, and the target organ present different forces and physical features that could alter potency via mechanotransduction pathways such as those described in the present study. Heterogeneity in the MSC population and donor variability are persistent challenges in clinical use of MSCs [71]. The present study identifies molecular rationale for how mechanical preconditioning could be utilized to improve therapeutic efficacy of MSCs in clinical applications and has revealed new possibilities for enhancement in the reparative potential of MSCs. Priorities for future studies will lie in understanding the role of biophysical cues in determination of the intracellular signaling that supports other mediators of MSC immune regulatory function, and whether aspects of mechanobiology will create further disparity in MSC performance or can be leveraged to produce greater consistency in MSC potency.

5. Conclusions

Collectively, our data support a role for fluid flow in modulation of the capacity of MSCs to suppress inflammatory response of the immune system. Force associated with flow promotes induction of COX2-PGE₂ signaling, which is essential for flow-enhanced potency in suppression of TNF- α inflammatory cytokine production by immune cells. Several mechanosensors are activated by flow in MSCs, but FAK is a chief regulator of COX2 expression and thus plays a critical role in MSC immunomodulatory activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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C.S.C. and P.L.W. are inventors on a patent for conditioning of stem and progenitor stem cells for cellular therapy (US 62/183,273).

Abbreviations

MSC	mesenchymal stromal cell
FAK	focal adhesion kinase
WSS	wall shear stress

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Fig. 1. WSS regulates COX2 and HO-1 expression independently of Ca^{2+} signaling

(A) Transcription of *PTGS*, *HMOX1*, *IL1RN* and *TSG6* is stimulated by WSS at 3 h and 6 h ($n = 4$ independent experiments; Kruskal-Wallis One Way ANOVA, *** $P < 0.001$). (B) WSS triggers elevated levels of Ca^{2+} concentration ($n = 4$ independent experiments, > 3 replicates per experiment). WSS was initiated 5 s after image acquisition began. See also Supplementary Video 1. (C) Quantification of Fluo-4 AM intensity by MetaMorph software captures multiple spikes in calcium flux following application of WSS. Pastel traces represent Ca^{2+} levels in individual cells ($n = 30$ cells); whereas, bold traces (blue or red)

represent the average intensity of values collected from individual cells. Note that y-axes are different scales to show small changes in static cultures. (D, E) WSS induces COX2 by 6 h WSS, which persists with 10 μ M of BAPTA-AM treatment. BAPTA-AM significantly reduces expression of HO-1 and TSG-6 ($n = 3$, Two Way ANOVA with Holm-Sidak multiple comparisons, $*P < 0.05$, $**P < 0.01$). All data are represented as mean $\pm$ SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. WSS-induced Akt activity is not responsible for COX2 increase

(A, B) Phosphorylation of Akt and ERK occurs rapidly in response to flow ($n = 3$, One Way ANOVA with Holm-Sidak multiple comparisons, $*P < 0.05$). (C, D) Treatment with the PI3K inhibitor LY294002 at 10 μ M fails to block COX2 increase in WSS-treated MSCs ($n = 3$, Kruskal Wallis ANOVA with Tukey multiple comparisons, $*P < 0.05$). All data are represented as mean $\pm$ SEM.

Fig. 3. FAK mediates induction of COX2 by flow

(A) Phosphorylated FAK-Y397 is detectable as prominent clusters after exposure to flow. Scale bar represents 50 μ m. (B) Quantification of pFAK-Y397 photomicrographs reveals increased clustering and signal intensity following WSS, suggesting that pFAK subcellular localization is altered (Kruskal Wallis ANOVA with Tukey multiple comparisons, $*P < 0.05$). (C, D) The FAK inhibitor PF-5662271 (10 μ M) blocked FAK phosphorylation ($n = 3$, Two Way ANOVA with Holm-Sidak multiple comparisons, $***P < 0.001$). FAK inhibition also impaired the ability of WSS to elevate COX2 and HO-1 protein levels at 6 h ($n = 4$,

unpaired *t*-test, **P* < 0.05). Cells cultured in suspension for 1 h (labeled as Susp or S) displayed reduction in pFAK and COX2 relative to static cultured cells treated with vehicle (*n* = 3, unpaired *t*-test, ****P* < 0.001). All data are represented as mean ± SEM.

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Fig. 4. Inflammatory cytokine suppression by MSCs requires FAK-dependent COX2-PGE₂ signaling

(A) PGE₂-EP_{2/4} receptor signaling determines MSC potency in suppression of inflammatory cytokine expression by LPS-stimulated immune cells from murine spleen. TNF- α suppression is normalized to fold reduction relative to fully activated splenocytes not placed in co-culture with MSCs. Treatment of splenocyte cultures with agonists of EP₂/EP₄ PGE₂ receptors, static cultured MSCs, or WSS-cultured MSCs significantly reduced the ability of activated immune cells to secrete TNF- α . Two Way ANOVA, ***P < 0.001. Comparison between static and WSS cultures treated with vehicle control revealed enhanced

immunomodulatory activity following transient exposure to shear stress ($n = 6$, Mann-Whitney Rank Sum test, $*P < 0.05$). Inhibition of FAK (PF-562271), EP₂ (PF-04418948), or EP₄ (L-161,982) signaling blocked WSS enhancement in MSC function. All data are represented as mean $\pm$ SEM. (B) WSS enhances the immunomodulatory properties of MSCs via a focal adhesion-dependent pathway. Schematic depicts a working model of mechanotransduction downstream of WSS which includes activation of FAK at focal adhesions. Phosphorylated FAK stimulates MSC anti-inflammatory activity by intermediate transcription factor(s) that transactivate *PTGS2*. Ca²⁺ influx and activation of PI3K/Akt and MAPK could contribute to MSC immune regulatory function, though these signaling mechanisms remain largely unstudied.