Inhibition of focal adhesion turnover prevents osteoblastic
differentiation through β-catenin mediated transduction of
pro-osteogenic substrate

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Abstract
The mechanism by which substrate surface characteristics are transduced by osteoblastic cells and their progenitors is not fully known. Data from previous studies by our group suggest the involvement of β-catenin in the mechanism by which substrate surface characteristics are transduced. This focal adhesion and β-catenin mediated mechanism functions through the liberation of β-catenin from focal adhesion complexes in response to pro-osteogenic substrate (POS) characteristics. After liberation, β-catenin translocates and facilitates upregulation of genes associated with osteogenesis. It is not known whether the observed correlation between focal adhesion turnover and β-catenin translocation directly results from focal adhesion turnover. In this study we inhibited focal adhesion turnover using a focal adhesion kinase inhibitor PF-573228. We found that inhibition of focal adhesion turnover resulted in an abrogation of the more rapid translocation and increased transcriptional activity of β-catenin induced by POS. In addition, inhibition of focal adhesion turnover mitigated the increase in osteoblastic differentiation induced by a POS as measured by alkaline phosphatase enzymatic activity and osteogenic gene and protein expression. Together, these data, coupled with previous findings, suggest that the observed β-catenin translocation is a result of focal adhesion turnover, providing evidence for a focal adhesion initiated, β-catenin mediated mechanism of substrate surface signal transduction.

KEYWORDS
bone, regenerative medicine, tissue engineering

1 | INTRODUCTION

Ongoing development of biomaterials for use in orthopedics and bone tissue engineering has yielded promising results through the modification of substrate surface characteristics. Surface modifications typically include grit blasting, acid etching, anodization, and plasma spraying with either metal or osteoconductive materials such as hydroxyapatite. These modifications aim to increase surface roughness, wettability, and protein absorbance. However, studies investigating biomaterial characteristics have not come to a consensus as to how substrate surface characteristics are transduced by bone cells and their progenitors. Such information would allow for optimization...
of biomaterial characteristics for osteogenesis and osteointegration. Focal adhesions are well-known mechanosensors, regulating expression of force and load-responsive transcription factors in osteoblastic cells in response to changes in external stimuli. Previous studies suggest changes in focal adhesions and changes in the cytoskeleton may at least partly contribute to the mechanism by which substrate characteristics are transduced.\textsuperscript{3,6}

Focal adhesion complexes are located at the membrane of the cell and bridge the gap between the extracellular matrix (ECM) and the intracellular environment.\textsuperscript{7} Cell signaling from the ECM to the intracellular compartment is mediated by focal adhesions and other transmembrane cell receptors, such as cadherins.\textsuperscript{8-10} Various cell pathways including matrix assembly, cell migration, and spatially organized signaling are regulated by these adhesions.\textsuperscript{11,12} Focal adhesions also share many proteins, including vinculin and α-catenin, with cadherins and bind the actin cytoskeleton in a manner similar to cadherins.\textsuperscript{13} Interestingly, one of the proteins that localizes at cadherins is β-catenin.\textsuperscript{13} At cadherin complexes β-catenin stabilizes the β-catenin and vinculin complex to allow binding to the actin cytoskeleton\textsuperscript{14,15} and this may also occur at focal adhesion complexes.

β-catenin also plays a crucial role in bone formation and maturation. The accumulation of β-catenin in the cytoplasm results in translocation to the nucleus where it regulates pro-osteoblastic gene expression.\textsuperscript{15} The importance of β-catenin in the promotion of osteogenesis and bone development is highlighted in a study by Case et al.\textsuperscript{16} They observed that β-catenin-deficient bone cells developed poor skeletal structures. Further, treatment with lithium chloride, which prevents inactivation of β-catenin, enhanced the response of bone cells to mechanical signals.\textsuperscript{17} These results highlight the vital role β-catenin plays in bone formation.

Focal adhesion kinase (FAK) activation is also a prominent signaling mechanism that, via ECM-integrin interactions, enhances the Wnt/β-catenin signaling pathway in bone cells.\textsuperscript{16} These data suggest a relationship between focal adhesions, FAK, and β-catenin-transcription mediated mechanical activation of osteogenesis. However, despite the extensive research on FAK and β-catenin's role in transduction of pro-osteogenic substrate(s) (POS), the mechanism by which the transduction occurs is still unknown.\textsuperscript{16}

To better understand this mechanism, it is essential to understand the interaction between substrate surface characteristics and their resulting effect on cell FA turnover and β-catenin translocation. A previous study by our group characterized substrates that combined polycaprolactone (PCL) and varying wt./wt. concentrations of hydroxyapatite particles (HAp).\textsuperscript{18} We determined that a 30% HAp/PCL substrate, referred to herein as the POS, induced significantly greater osteoblastic differentiation, greater focal adhesion maturation, and more rapid focal adhesion turnover compared to all other substrates examined. Interestingly, a more rapid translocation of β-catenin from the membrane-bound protein fraction to the nucleus also occurred.\textsuperscript{18} This led us to hypothesize a novel mechanism by which substrate surface characteristics are transduced by osteoblastic cells. We hypothesized that the mechanism by which this occurs involves the liberation of β-catenin from focal adhesion complexes, located at the cell membrane. More osteogenic substrates cause greater focal adhesion turnover which results in greater liberation of β-catenin, that in turn allows for greater translocation of β-catenin and an increase in osteoblastic differentiation. This study seeks to evaluate if the more rapid translocation of β-catenin is a consequence of the observed focal adhesion turnover. We hypothesized that inhibition of focal adhesion turnover would result in a decrease in osteoblastic differentiation, a delay in the translocation of β-catenin from the membrane-bound protein fraction to the nucleus, and a decrease in nuclear activity in cells cultured on POS compared to cells cultured on POS without FAK inhibition.

To evaluate this hypothesis, we examined the effect of a focal adhesion inhibitor, PF-573228, which is known to inhibit focal adhesion turnover in vitro,\textsuperscript{18} on osteoblastic differentiation, focal adhesion turnover and maturation, and β-catenin localization and activity over time on a previously examined POS.\textsuperscript{19,20} We observed that cells cultured on the POS displayed increased osteoblastic differentiation, greater focal adhesion maturation, a more rapid (24 h) translocation of β-catenin from the membrane-bound fraction to the nuclear fraction, and greater nuclear activity compared to cells cultured on tissue culture polystyrene (TCPS), corroborating previous findings.\textsuperscript{16,21,22} However, upon addition of the FAK inhibitor, focal adhesion turnover was significantly inhibited, the rapid translocation of β-catenin was prevented, and osteoblastic differentiation was significantly downregulated. Indeed, cells cultured on POS in the absence of FAK inhibitor more closely mimicked cells cultured on TCPS in the absence of FAK inhibition.

2 | MATERIALS AND METHODS

2.1 | Materials

All chemicals used for nanoparticle and substrate synthesis were obtained from Sigma-Aldrich (St. Louis, MO).

2.2 | Substrate synthesis

2.2.1 | PCL and hydroxyapatite substrate synthesis

To create a POS, we synthesized a substrate composed of PCL and HAp in a ratio that was determined previously to be optimally osteogenic.\textsuperscript{18} Briefly, 5000 molecular weight PCL was suspended at a 2.5% wt./vol. ratio in chloroform. The mixture was then homogenized until all PCL was dissolved into solution. After homogenization, 500 nm HAp was added to the solution at a ratio of 30% wt./wt. with the PCL. Using a dip-coating technique, 22 mm coverslips were coated and allowed to dry at room temperature for 24 h. Prior to use all substrates were submerged in 70% ethanol for 5 min before the ethanol was aspirated and samples allowed to air dry. After drying samples were exposed to ultraviolet light for 1 h.
2.3 | Cell characterization

2.3.1 | Osteoblast cell culture

To assess osteogenesis, the differentiation capacity of human fetal osteoblasts (hFOB 1.19 cells), a preosteoblastic cell line, was assessed as previously described. Briefly, for proliferation of hFOB 1.19 cells, cells were cultured at 33.5°C with 5% CO₂ to 80% confluence in standard DMEM:F12 media supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD) and 1% Penicillin/Streptomycin mixture (Sigma, St. Louis, MO). To induce differentiation of the hFOB 1.19 cells, the standard media was supplemented with 100 μg/ml ascorbic acid, 10⁻⁸ M menadione, and 10⁻⁸ M dihydroxy-vitamin D3 (Sigma, St. Louis, MO), and the cells were cultured at 39.5°C with 5% CO₂ until the desired timepoint. In some experiments the FAK inhibitor, PF-573228, which is known to inhibit focal adhesion turnover in vitro, was suspended in dimethyl sulfoxide (DMSO), and added to differentiation media at a molar concentration of 200 nM. This concentration was determined based on levels of FAK phosphorylation activity at varying FAK inhibitor concentrations (Figure S1). In addition, an identical amount of DMSO without FAK inhibitor was added to control media. Media was changed every 3 days unless otherwise stated for all experimental methods.

2.3.2 | Alkaline phosphatase (AP) activity

hFOB 1.19 cells were cultured on either TCPS or the POS in either the presence or absence of the FAK inhibitor PF-573228 at 15,000 cells/cm² and cultured under differentiation conditions for 7 days. Differentiation was evaluated using a colorimetric AP enzymatic activity assay as previously described. Briefly, cells were freeze thawed in 400 μl of 0.05% Triton X-100 in phosphate buffered saline twice and then the cell lysate collected. 10 μl of each sample lysate was removed and used to quantify total protein concentration using a Pierce BCA protein assay kit (Thermo Fisher, Waltham, MA). AP enzymatic activity was then determined by conversion of p-nitrophenol phosphate to p-nitrophenol. 200 μl of AP reaction buffer was then added to each sample and incubated at room temperature for 30 min. After incubation 50 μl of each sample was placed in 200 μl of 0.1 NaOH in a 96 well-plate to quench the reaction. All samples were then assessed at 410 nm, and SIGMA units calculated based on the standard curve. All readings were then normalized to total protein concentration to control for variations in cell number between samples.

2.3.3 | Osteoblastic gene expression

hFOB 1.19 cells were seeded on TCPS or the POS in either the presence or absence of the FAK inhibitor PF-573228 at 100,000 cells/cm² and cultured under differentiation conditions for 1 and 7 days. At each time point RNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitative real time PCR (RT-qPCR) was performed using a C1000 Touch Thermal Cycler with CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA). PowerUp Sybr Green Master Mix was used to quantify gene expression (Thermo Fisher Scientific). The genes we evaluated are associated with the various stages of osteoblastic differentiation and are as follows; alkaline phosphatase (ALPL), Osteocalcin (BGLAP), Collagen1-A1 (COL1A1), Runt related transcription factor-2 (RUNX2), and Sp7 Transcription Factor (SP7). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as a reference gene for all samples. All primers used in this study were PrimePCR Sybr Green Assay Primers (Bio-Rad Laboratories, Hercules, CA) and the unique assay ID numbers are provided in Table S1. The ΔΔCt method was used to quantify fold-change in gene expression relative to the housekeeping gene GAPDH, as previously described.

2.3.4 | Osteoblastic protein expression

hFOB 1.19 cells were seeded on TCPS or the POS in either the presence or absence of the FAK inhibitor PF-573228 at 100,000 cells/cm² and cultured under differentiation conditions for 1, 3, and 7 days. At each time point cells were lysed and the lysate was collected. The proteins we evaluated are associated with the various stages of osteoblastic differentiation and are as follows; Collagen1-a1, Osteocalcin, and Osteopontin. 10 μl of cell lysate from each sample at the various timepoints was removed and used to quantify total protein concentration using a Pierce BCA protein assay kit (Thermo Fisher, Waltham, MA). 25 μg of total protein from each sample at the various time points was then used to quantify Protein expression using a either a Human Collagen1-a1, Human Osteocalcin, or Human Osteopontin DuoSet ELISA (R&D Biosystems, Minneapolis, MN). All samples were normalized to total protein concentration and results quantified.

2.3.5 | FAK activity

hFOB 1.19 cells were cultured on either TCPS or the POS at 100,000 cells/cm² under differentiation conditions for 12, 24, 48, 72, or 96 h. At each time point protein was harvested as previously described. Briefly, 400 μl of a solution of 1× RIPA buffer and protease inhibitor cocktail (Thermo Fisher, Waltham, MA) was placed into each well for 15 min on ice. After 15 min, the wells were scrapped using a cell scraper and the cell slurry was then moved to a 1.5 ml conical tube. The cells were then lysed further by pipetting cells 10 times through a 21-gauge needle. Cells were then centrifuged at 13,000 RPM for 20 min and the supernatant harvested. Cell lysate was then analyzed for focal adhesion kinase activity using FAK (Total) Human ELISA Kit and FAK (Phospho) [pY397] Human ELISA Kit (Thermo Fisher, Waltham, MA). Phosphorylated FAK was normalized to total FAK in each sample.
2.3.6 | Focal adhesion staining

hFOB 1.19 cells were cultured on TCPS or the POS in either the presence or absence of the FAK inhibitor PF-573228 at 15,000 cells/cm² under differentiation conditions for 12, 24, 48, 72, or 96 h. At the selected time point, cells were stained using ab196454, an anti-vinculin antibody conjugated to AlexaFluor 488 (Abcam, Cambridge, UK). Briefly, cell culture medium was removed, and cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After fixation, cells were washed twice with wash buffer consisting of 0.05% Tween-20 in PBS. Cells were then permeabilized with 0.1% Triton-X100 in PBS for 5 min. After permeabilization, cells were washed twice with wash buffer and then blocked for 1 h at room temperature in Odyssey Blocking Buffer (Thermo Fisher Scientific, Waltham, MA). After blocking anti-vinculin antibody was diluted to 1:250 in blocking buffer and incubated overnight at room temperature. Cells were then washed three times in wash buffer. DAPI stain, diluted at 1:1000 in PBS, was added and incubated at room temperature for 5 min. After incubation samples were mounted on glass slides using ProGold Anti-fade Mounting Solution (Thermo Fisher Scientific, Waltham, MA). Representative images for each group and timepoint are shown in Figure S2.

2.3.7 | Focal adhesion characterization

Confocal images of the various samples were gathered using an LSM 710 Confocal Microscope (Zeiss, Jena, Germany). Using a modified version of custom MATLAB-based software which can be found at www.lemmonlab.com, images were analyzed for focal adhesion size and morphology, and number normalized to cell number. This software segregates pixels that contain fluorescence from those that do not and then measures the length and width of each focal adhesion. A contrast threshold of 0.450 was selected and applied to all images allowing segmentation of the individual focal adhesions. If the segmentation did not accurately represent the original image, a different threshold value was chosen manually until segmentation of the focal adhesions was representative of the original images. Thresholding analysis was then performed and focal adhesion number, length, and width dimensions (µm), and area (µm²) were output and recorded.

2.3.8 | GTPase activity

hFOB 1.19 cells were cultured on the POS at 100,000 cells/cm² under differentiation conditions for 4, 12, 24, 48, and 72 h. After culture, cells were lysed in accordance with the protocol from the G-LISA Activation Assay Kit (Cytoskeleton Inc., Denver, CO) for RhoA, Rac1, or Cd42. 10 µl of each sample lysate was removed and used to quantify total protein concentration using Pierce BCA protein assay kit (Thermo Fisher, Waltham, MA). Cell lysates were then examined using either the RhoA, Rac1, or Cd42 G-LISA activation assay kits as per the designated protocol and GTPase activity quantified. All cell fractions were normalized to total protein content.

2.3.9 | β-catenin cellular dynamics

hFOB 1.19 cells were cultured on TCPS or the POS in either the presence or absence of the FAK inhibitor PF-573228 at 100,000 cells/cm² under differentiation conditions for 4, 12, 24, 48, 72, or 96 h. After culture, cells underwent protein fractionation using the Subcellular Protein Fractionation kit for Cultured Cells (Thermo Fisher Scientific, Waltham, MA). After fractionation, 10 µl of cell lysate from each fraction was removed and used to quantify total protein concentration using a Pierce BCA protein assay kit (Thermo Fisher, Waltham, MA). 25 µg of total protein from each cell fraction at the various time points was then used to quantify total β-catenin protein concentration in the various cell fractions over time using a Human Total β-catenin DuoSet IC ELISA (R&D Biosystems, Minneapolis, MN). All cell fractions were then normalized to total β-catenin concentration at the respective timepoints.

2.3.10 | Luciferase based β-catenin translocation reporter assay

To validate nuclear β-catenin translocation and activity from cell fractionation experiments, hFOB 1.19 cells were examined using a luciferase-based reporter assay (Promega Corporation, Madison, WI) as previously described. Briefly, hFOB1.19 cells were transiently transfected with either a TOPFLASH reporter construct, which contains a firefly luciferase reporter that is activated by TCF/LEF binding, or a FOPFLASH reporter construct, which has a mutated TCF/LEF protein that prevents β-catenin activation of the firefly reporter. After 24 h, 100,000/cm² cells were seeded onto either TCPS or the POS in the presence or absence of the FAK inhibitor, PF-573228, and cultured for 12, 24, 48, 72, or 96 h. After culture, the cells were lysed and a TOPFLASH/FOPFLASH quantified. β-catenin translocation and activity in cells on TCPS or the POS in the presence or absence of the FAK inhibitor was then calculated.

2.4 | Statistical analysis

AP expression was assessed for 4 samples with each sample being the average of two replicates. Gene expression was assessed for 4 samples with each sample being the average of two replicates. Protein expression was assessed for 3–4 samples with each sample being the average of 3 replicates. FAK activity was assessed for 6 samples. Focal adhesion quantification and characterization was assessed with 3 samples with each sample being the average of 2 to 3 replicates. Each image consists of approximately 5 to 50 cells or roughly 500 to 5000 focal adhesions. β-catenin localization was assessed for 3 to 4 samples with each sample consisting of two replicates. Changes in AP activity
were assessed using two-way ANOVA followed by Tukey’s post-hoc analysis and are reported as mean ± SD. Changes in osteoblastic gene expression were assessed using two-way ANOVA followed by Tukey’s post-hoc analysis and are reported as mean ± SD. FAK kinase activity was assessed using an unpaired t-test followed by Bonferroni post-hoc analysis and are reported as mean ± SD. Changes in focal adhesion number, size, and eccentricity were assessed using two-way ANOVA followed by Tukey’s post-hoc analysis and are reported as mean ± SD. All analysis was performed using GraphPad Prism version 8.1.1 for Windows (GraphPad Software, La Jolla, California, USA). Values of \( p < .05 \) were considered statistically significant.

3 | RESULTS

3.1 Osteoblastic differentiation and FAK activity are increased in cells cultured on POS in the absence of FAK inhibitor

We first corroborated previous findings which demonstrated that cells cultured on POS display greater osteoblastic differentiation than cells cultured on TCPS. hFOB1.19 cells were cultured on TCPS or POS in the absence of FAK inhibitor and AP enzymatic activity quantified at 1, 3, and 7 days (Figure 1). After 1 day in culture, cells cultured on POS exhibited a significant 1-fold increase in AP enzymatic activity compared to cells cultured on TCPS. After 3 and 7 days in culture, cells cultured on POS again exhibited a significantly greater 2-fold and 1-fold increase in AP enzymatic activity, respectively, compared to cells cultured on TCPS. Moreover, the total increase in AP enzymatic activity from day 1 to day 7 was greater in cells cultured on POS (0.069 U/μG) compared to cells cultured on TCPS (0.039 U/μG) suggesting greater osteoblastic differentiation in cells culture on POS overall.

We then assessed gene expression of key osteoblastic differentiation markers after 1 and 7 days in culture in cells cultured on TCPS or POS in the absence of FAK inhibitor (Figure 2). At day 1 no significant differences were observed between cells cultured on TCPS and cells cultured on POS in the absence of FAK inhibitor. At day 7, gene expression of ALPL, COL1A1, RUNX2 was significantly increased in cells cultured on POS compared to cells cultured on TCPS in the absence of FAK inhibitor, similar to what has been observed previously. The roughly 1-fold increase in expression observed in ALPL, BGLAP, and RUNX2 also suggests a greater maturation of osteoblasts cultured on POS compared to those cultured on TCPS, as these markers are indicative of mature osteoblast differentiation.

To determine if the observed changes in gene expression correlated to changes in protein expression, we examined the expression of key proteins indicative of osteoblastic differentiation in cells cultured on TCPS and POS in the absence of FAK inhibitor (Figure 3). At day 1, no significant differences, in any protein examined, was observed between cells cultured on TCPS and cells cultured on POS. At day 3 however, cells cultured on POS exhibited a 3-fold increase in the COL1A1 protein expression (Figure 3), a marker of early commitment to the osteoblastic lineage. Neither Osteocalcin (OCN) nor Osteopontin (OPN) were significantly regulated at day 3 between cells cultured on POS and cells cultured on TCPS. At day 7 all proteins examined were significantly upregulated in cells cultured on POS compared to cells cultured on TCPS. Cells cultured on POS in the absence of FAK inhibitor exhibited a roughly 1-fold increase in both OCN (Figure 3) and OPN (Figure 3), both indicative of mature osteoblastic differentiation, and a 0.5-fold increase in COL1A1 protein expression compared to cells cultured on TCPS. These data coupled with both the AP enzymatic activity and gene expression data confirm that cells cultured on the POS differentiate significantly more compared to cells cultured on TCPS, as previously observed.16

After confirming that POS induced significantly greater osteoblastic differentiation compared to TCPS, we then examined FAK activity (Figure 4). Normalized FAK activity was quantified by examining the concentration of phosphorylated FAK divided by total concentration of FAK over time in cells cultured on TCPS and cells cultured on POS in the absence of FAK inhibitor. We observed significant increases in normalized FAK phosphorylation at 24 h in cells cultured on POS compared to cells cultured on TCPS. The observed increase remained until 72 h, where normalized FAK phosphorylation then decreased and was significantly less in cells cultured on POS relative to cells cultured on TCPS. Interestingly, cells cultured on POS and TCPS followed a similar trend. Normalized FAK phosphorylation over time increased and remained elevated for 48 h before returning to basal level, with the POS inducing a more rapid increase in FAK phosphorylation. Interestingly, activation levels of Rac1, Cd42, and RhoA,
GTPases, which participate in actin cytoskeleton and focal adhesion formation, were unchanged over the course of 72 h (Figure S3).

### 3.2 Inhibition of focal adhesion turnover prevents POS induced increase in osteoblastic differentiation

To examine the role of focal adhesion turnover in the proposed mechanism, we exposed cells to the FAK inhibitor, PF-573228, that prevents focal adhesion turnover in vitro. To confirm the concentration of inhibitor necessary to prevent FAK phosphorylation we examined FAK phosphorylation using ELISA at 3 different concentrations (100, 150, and 200 nM) in cells cultured on TCPS (Figure S1). We observed significant decreases in normalized FAK phosphorylation at a 200 nM concentration and therefore 200 nM was used for all following experiments.

To evaluate changes in focal adhesion turnover and maturation, we first examined how introduction of FAK inhibitor to cells cultured on POS would affect focal adhesion number, area, and eccentricity over time. We observed that from 4 to 12 h after seeding in cells cultured on POS in the absence of FAK inhibitor that a significant increase (approximately 100 adhesions per cell) in focal adhesion number occurred (Figure 5A). This number then decreased over the next 36 h to roughly 75 adhesions per cell before again increasing to approximately 150 adhesions/cell. As expected, this cyclical pattern was not observed in cells cultured on POS in the presence of FAK inhibitor where the number of focal adhesions over time remained relatively stable at 100 adhesions per cell. In addition, cells cultured on POS in the absence of FAK inhibitor became significantly larger between 48 and 96 h, increasing in size roughly 10 μm (Figure 5B). This increase in area was not observed in cells cultured on POS in the presence of FAK inhibitor, with significantly smaller adhesions being present at 72 and 96 h after cell seeding compared to cells cultured in the absence of FAK inhibitor. Moreover, as previously reported, adhesion eccentricity decreased (became more circular) in cells cultured on POS in the absence of FAK inhibitor at 72 and 96 h after cell seeding compared to cells cultured in the presence of FAK inhibitor (Figure 5C). These data suggest that the FAK inhibitor was preventing focal adhesion turnover and maturation over time, as typically resulted from culturing of cells on the POS.

Following the observation that FAK inhibitor was in fact, preventing focal adhesion turnover and maturation, we then wanted to examine how the introduction of FAK inhibitor to cells cultured on POS affected osteoblastic differentiation. We observed, in cells cultured in the presence of FAK inhibitor on POS, a significant reduction in AP enzymatic activity at all timepoints compared to cells cultured on POS in the absence of FAK inhibitor (Figure 1). In addition, AP enzymatic activity was not significantly different in cells cultured on POS in the presence of FAK inhibitor and cells cultured on TCPS in the absence of the inhibitor at days 1 and 3 but did result in a significant reduction in AP activity at day 7.

![Figure 2](attachment:image_url)

**Figure 2** Osteoblastic gene expression on tissue culture polystyrene (TCPS) or pro-osteogenic substrate (POS) in the presence (+) or absence (−) of focal adhesion kinase (FAK) inhibitor PF-573228. Evaluation of osteoblastic gene expression of (A) alkaline phosphatase (ALPL), (B) osteocalcin (BGLAP), (C) collagen1-α1 (COL1A1), (D) RUNX2, and (E) osterix (SP7) at 1 and 7 days in culture. Significance is considered $p < 0.05$ only within the same timepoint. *, significantly different than all other groups; $\star$, significantly different than TCPS−; $\star\star$, significantly different than pro-osteogenic−. n = 4 samples with each sample being the average of two replicates.
We next examined osteoblastic gene expression in the presence and absence of FAK inhibitor. At day 1 no differences were observed in cells cultured on TCPS or POS regardless of the presence or absence of FAK inhibitor. However, at day 7 gene expression for ALPL, OCN, RUNX2, and SP7 in cells cultured in the presence of FAK inhibitor on the POS was significantly reduced compared to cells cultured on POS in the absence of FAK inhibitor. Unlike all other genes examined, COL1A1 gene expression was unchanged in cells cultured on both TCPS and POS in the presence of FAK inhibitor at day 7 maintaining expression similar to, if not slightly greater, than the expression observed in cells cultured on the same substrate in the absence of FAK inhibitor.

We also examined expression of key osteoblastic proteins over time in cells cultured on TCPS or POS in the presence and absence of FAK inhibitor (Figure 3). At day 1 we saw no significant changes in protein expression across all groups regardless of the substrate or FAK inhibitor used during cell culture. In cells cultured on POS in the presence of FAK inhibitor compared to cells cultured on POS in the absence of FAK inhibitor at day 3 we observed a significant 3-fold, 3-fold, and 4-fold reduction of COL1A1, OCN, and OPN protein expression, respectively. In cells cultured on TCPS, only OPN was significantly downregulated by the presence of FAK inhibitor at day 3. At day 7, similar to what we observed in osteoblastic gene expression, we observed a significant 4-fold and 3-fold reduction in OCN and OPN, respectively, in cells culture on POS in the presence of FAK inhibitor.

**FIGURE 3** Osteoblastic protein expression on tissue culture polystyrene (TCPS) or pro-osteogenic substrate (POS) in the presence (+) or absence (−) of focal adhesion kinase (FAK) inhibitor PF-573228. Evaluation of normalized expression of proteins related to osteoblastic differentiation (A) collagen1-a1 (COL1A1), (B) osteocalcin (OCN), and (C) osteopontin (OPN) at 1, 3 and 7 days in culture. Significance is considered *p* < 0.05 only within the same timepoint. *, significantly different than all other groups; ★, significantly different than TCPS +; x, significantly different than pro-osteogenic −. n = 3–4 samples with each sample being the average of three replicates.

**FIGURE 4** Focal adhesion kinase activity over 96 h in hFOB 1.19 cells cultured on either tissue culture polystyrene (TCPS) or pro-osteogenic substrate. Significance (*) is considered *p* < 0.05 within the same timepoint. n = 6 samples.

We next examined osteoblastic gene expression in the presence and absence of FAK inhibitor.
inhibitor compared to cells cultured on POS in the absence of FAK inhibitor. We observed similar results in cells cultured on TCPS in the presence of FAK inhibitor at day 7, with a significant reduction in protein expression in both OCN and OPN compared to cells cultured in the absence of FAK inhibitor. Interestingly, conversely to what was observed in gene expression, COL1A1 protein expression was significantly downregulated (4-fold) in cells cultured on POS in the presence of FAK inhibitor compared to cells cultured on POS in the absence of FAK inhibitor.

3.3 | Rapid β-catenin translocation and nuclear activity are reduced by FAK inhibition

To evaluate how inhibiting FAK turnover affects β-catenin localization and activity over time we examined β-catenin localization in cells on POS and TCPS in the presence and absence of FAK inhibitor (Figure 6). We observed, in the absence of FAK inhibitor, a more rapid (24 h) translocation of β-catenin from the membrane bound fraction to the nucleus in cells cultured on POS compared to cells cultured on TCPS. In cells cultured on POS in the absence of FAK inhibitor β-catenin concentration was significantly increased in the membrane fraction 48 h after cell seeding while nuclear β-catenin concentration remained low (Figure 6A,B). Seventy two hours after seeding, β-catenin concentration within the membrane bound fraction decreased while β-catenin concentration in the nuclear fraction significantly increased before the concentration of β-catenin in both the membrane and nuclear fraction returned to basal levels at 96 h. Cells cultured on TCPS in the absence of FAK inhibitor followed an identical trend of β-catenin translocation, although the process was delayed roughly 24 h, beginning at 72 h. We corroborated the observed β-catenin translocation using a luciferase-based reporter assay and observed a similar 24-h delay in the nuclear translocation of β-catenin in cells cultured on TCPS in the absence of FAK inhibitor compared to cells cultured in the absence of FAK inhibitor on POS. In addition, nuclear activity was significantly greater at 24, 48, and 72 h in cells cultured on POS in the absence of FAK inhibitor compared to cells cultured in the absence of FAK inhibitor on TCPS (Figure 6C). This is also indicative of the previously observed increases in osteoblastic differentiation, as nuclear activity of β-catenin is known to upregulated various osteogenic pathways such as the Wnt signaling pathway.24

Conversely, addition of FAK inhibitor to cells cultured on POS resulted in a significant reduction in β-catenin concentration and rate of translocation compared to cells cultured in the absence of FAK inhibitor. A 3-fold reduction β-catenin concentration in the membrane-bound fraction β-catenin (Figure 6A) and a roughly 1.5-fold reduction in β-catenin concentration within the nuclear fraction was observed at 48 h in cells cultured on POS in the presence of FAK inhibitor compared to cells cultured on POS without FAK inhibitor (Figure 6B). Interestingly, the addition of FAK inhibitor to cells cultured on TCPS did not significantly reduce the β-catenin concentration within the membrane-bound fraction at 48 h, although it was decreased. β-catenin concentration in the nuclear fraction was unchanged at 48 h in cells cultured on TCPS in the absence of FAK
inhibitor compared to cells cultured on TCPS or POS in the presence of FAK inhibitor. Seventy two hours after cell seeding, β-catenin concentration in the membrane-bound fraction was significantly increased in cells cultured on POS in the presence of FAK inhibitor compared to cells cultured on POS in the absence of FAK inhibitor. In the nuclear fraction, the presence of FAK inhibitor significantly reduced (5-fold) the concentration of β-catenin compared to cells cultured on POS in the absence of FAK inhibitor. β-catenin concentration in the membrane-bound fraction of cells cultured on POS in the presence of FAK inhibitor was significantly reduced (5-fold) compared to cells cultured on TCPS in the absence of FAK inhibitor. In the nuclear fraction at 72 h, β-catenin concentration was not significantly different in cells cultured on TCPS in the absence of FAK inhibitor compared to cells cultured on POS or TCPS in the presence of FAK inhibitor. At 96 h no significant changes in β-catenin concentration were observed in the membrane bound-fraction between any of the groups. In the nuclear fraction, cells cultured on POS in the presence of FAK inhibitor had a significantly greater β-catenin concentration compared to cells cultured on POS in the absence of FAK inhibitor and cells cultured on TCPS in the presence of FAK inhibitor. In cells cultured on TCPS in the absence of FAK inhibitor we observed significantly 4-fold, 4.5-fold, and 1-fold increase in β-catenin concentration compared to TCPS in the presence of FAK inhibitor, POS in the absence of FAK inhibitor, and POS in the presence of FAK inhibitor respectively. Interestingly, introduction of FAK inhibitor to cells cultured on POS resulted in a 24 h delay more similar to that of cells cultured on TCPS in the absence of FAK inhibitor compared to cells cultured on POS in the absence of FAK inhibitor.

We again corroborated the observed β-catenin translocation with a luciferase-based reporter assay. We observed similar trends to those observed previously. Nuclear activity in cells cultured on POS in the absence of FAK inhibitor was significantly greater than all other groups at 24, 48, and 72 h (Figure 6C). At 48 h, nuclear activity was significantly greater in cells cultured on TCPS in the absence and cells cultured in POS in the absence of FAK inhibitor compared to cells cultured on POS in the absence of FAK inhibitor. At 96 h, cells cultured

FIGURE 6 β-catenin localization, total β-catenin concentration, and β-catenin transcriptional activity over 96 h in hFOB 1.19 cells cultured on either tissue culture polystyrene (TCPS) or pro-osteogenic substrate (POS) in the presence (+) or absence (−) of focal adhesion kinase (FAK) inhibitor PF-573228. Quantification of normalized β-catenin concentration in either the (A) membrane bound cell fraction, (B) nuclear cell fraction. (C) Nuclear β-catenin activity quantified by TOPFLASH activity. (D) Total β-catenin concentration. Significance is considered p < 0.05 only within the same timepoint. *, significantly different than all other groups; $, significantly different than TCPS−; ★, significantly different than TCPS+; x, significantly different than pro-osteogenic−. Membrane, nuclear, and total β-catenin, n = 4. Nuclear activity, n = 3–4 samples, with each sample being the average of 2 replicates.
on TCPS in the absence of FAK inhibitor displayed significantly greater nuclear activity compared to cells cultured on TCPS or POS in the absence of FAK inhibitor. However, this result was not corroborated by β-catenin concentration in the nuclear fraction, suggesting a difference between the localization of β-catenin and the nuclear activity.

Total β-catenin concentration varied significantly over time between the observed groups exhibiting the importance of normalizing all fractions to the total β-catenin concentration (Figure 6D). At 12 h cells cultured on POS in the absence of FAK inhibitor expressed greater amounts of β-catenin compared to cells cultured on TCPS in the presence and absence of FAK inhibitor. No differences were observed at 24 h between any of the groups. At 48 and 72 h cells cultured on POS in the absence of FAK inhibitor expressed significantly greater total β-catenin concentration compared to all other groups. Total β-catenin concentration was also significantly greater in cells cultured on TCPS in the absence of FAK inhibitor compared to cells cultured on TCPS and POS in the presence of FAK inhibitor. At 96 h, cells cultured on TCPS in the absence of FAK inhibitor expressed significantly greater total β-catenin concentration compared to all other groups.

4 | DISCUSSION

Determining a mechanism by which substrate surface characteristics are transduced by osteoblastic cells and their progenitors would provide avenues for further optimization of bone implants and novel biomaterials for use in bone tissue engineering. Previous research by our group determined that cells cultured on POS exhibit greater osteoblastic differentiation, focal adhesion maturation, and focal adhesion turnover.16 This turnover correlated with a more rapid translocation of β-catenin from the membrane bound fraction to the nuclear fraction.16 Upon inhibition of β-catenin binding to TCF/LEF, the observed increases in osteoblastic differentiation caused by the POS were abrogated.16 This data led us to propose a focal adhesion initiated, β-catenin mediated mechanism by which substrate surface characteristics are transduced. While previous results suggest that β-catenin is liberated from focal adhesions and then participates in osteogenic regulation after nuclear translocation, we were unable to determine conclusively if the translocation was a result of focal adhesion turnover. Through the inhibition of FAK, we were able to determine the role of focal adhesion turnover plays in the observed changes in β-catenin translocation, nuclear activity, and osteoblastic differentiation.

We hypothesized that inhibition of focal adhesion turnover in cells cultured on POS would result in a delay in the translocation of β-catenin from the membrane-bound protein fraction to the nucleus, a decrease in nuclear activity, and a decrease in subsequent osteoblastic differentiation compared to cells cultured on POS without FAK inhibition.

We observed, as previously reported25 in cells cultured on POS in the absence of FAK inhibitor that a significant increase in osteoblastic differentiation occurred as measured by increases in the expression of key osteoblastic genes (Figure 2) and proteins (Figure 3) as well as increases in AP activity (Figure 1) compared to cells cultured on TCPS. In addition, cells cultured on POS in the absence of FAK inhibitor exhibited greater focal adhesion activity, as measured by an increase in FAK phosphorylation, which suggests an increase in focal adhesion turnover compared to cells cultured on TCPS in the absence of FAK inhibitor, which was observed in a previous study by our group.25 Moreover, a more rapid (24 h) translocation of β-catenin from the membrane-bound protein fraction to the nucleus and greater nuclear activity was also observed (Figure 6). Studies by Xie et al. observed that in adipocyte-derived stem cells cultured on POS of increasing concentration of polydimethylsiloxane, commitment to the osteogenic lineage was increased.26 β-catenin nuclear translocation was also upregulated on POS of increasing stiffness.26 They also observed increased vinculin protein expression (~30%), suggesting greater focal adhesion formation in conjunction with the enhanced β-catenin nuclear translocation.26 Studies by Hamilton and Brunette came to a similar conclusion. FAK activity and focal adhesion formation were both upregulated on a grooved POS but were not observed on smooth non-POS.27 These studies suggest that focal adhesions and FAK are key mediators of substrate surface characteristics and help drive commitment to osteoblastic differentiation.

In the study presented here, addition of the FAK inhibitor to cells cultured on POS resulted in a decrease in the previous increases in osteoblastic differentiation resulting from culture on the POS (Figures 1–3). AP activity of cells cultured on POS in the presence of FAK inhibitor at day 7 more closely resembled AP activity of cells cultured on TCPS in the absence of FAK inhibitor, suggesting that inhibition of FAK may inhibit transduction of the POS. Again, Hamilton and Brunette observed a similar result.27 Upon inhibition of FAK phosphorylation, the previously observed increase in osteoblastic differentiation, as measured by RUNX2 nuclear translocation, on the grooved POS was inhibited.27 In addition, Sun et al. and Rajshankar et al. both reported that in mice lacking FAK in osteoblastic and pre-osteoblastic cells, a low bone mass phenotype and increased marrow adiposity was observed.28,29 This was attributed to decreased osteoblastic proliferation, differentiation, and mesenchymal commitment to the osteoblastic lineage, thus reaffirming the results presented here that FAK, and therefore focal adhesion formation and turnover regulate osteoblastic differentiation.28 It is worth noting, that in both cells cultured on POS or TCPS in the presence of FAK inhibitor, gene expression of ALPL, RUNX2, SP7 were significantly down regulated, however COL1A1 gene expression was not. COL1A1 gene expression was the same if not slightly elevated in cells on both substrates when FAK inhibitor was introduced. This observed increase was not mirrored in protein expression after 7 days, with COL1A1 protein significantly downregulated in cells cultured in the presence of FAK inhibitor compared to cells cultured in the absence of FAK inhibitor on POS. A previous study by Hori et al. reported similar protein expression findings in fibroblasts.25 They reported that increases in FAK (Tyr397) phosphorylation resulted in increases in collagen-I expression. Upon
We hypothesize that the increased COL1A1 may be a direct result of the lack of COL1A1 protein expression. Because the cell is unable to form a stable adhesion and because it cannot deposit collagen to form a stable ECM to adhere to, it upregulates gene expression COL1A1 in an attempt to compensate for the dysregulation caused by the addition of FAK inhibitor. Rajshankar et al. also postulated a similar hypothesis, as dysregulation of FAK in vivo resulted in significant collagen deficiencies, as well as poor bone phenotype because of decreased osteoblastic differentiation and bone formation.\textsuperscript{29}

β-catenin translocation was also significantly affected by the addition of FAK inhibitor. The more rapid translocation (12 h prior to TCPS) of β-catenin (Figure 6A,B), and the significant increase in nuclear transcriptional activity (Figure 6C) were no longer observed after the addition of FAK inhibitor. Instead, we observed that cells cultured on POS in the presence of FAK inhibitor appeared to also have the same 24 h delay in β-catenin translocation from the membrane-bound protein fraction to the nuclear fraction that was observed in cells cultured on TPCS in the absence of FAK inhibitor. Transcriptional activity of β-catenin in cells cultured on POS in the presence of FAK inhibitor mirrored the transcriptional activity of cells cultured on TCPS in the absence of FAK inhibitor almost identically. However, at 96 h, we observed a decrease in transcriptional activity in cells cultured on POS in the presence of FAK inhibitor compared to the increase observed in cells cultured in the absence of FAK inhibitor in on TCPS or POS. Taken together, data presented here, and previously,\textsuperscript{5,26–28} suggest that focal adhesion turnover plays a critical role in the observed increase in β-catenin translocation and transcriptional activity that results in increased osteoblastic differentiation in response to the POS. There was an increase in total β-catenin concentration at 48 and 72 h in cells cultured in the absence and presence of FAK inhibitor respectively. The work presented here suggests that the increase is likely due to a combination of β-catenin liberation from the cells as well as an increase in β-catenin production or decrease in phosphorylation of β-catenin in response to the POS, though more work is necessary to definitively say.

The study presented here and previously,\textsuperscript{5} also seeks to answer the question posed by Jamora and Fuchs. The question is as follows, it is known that disassociation of adhesion complexes occurs in response to various stimuli, but what happens to the proteins, such as β-catenin, that make up the adhesion complex after disassociation?\textsuperscript{30} Do they participate in other facets of cell mechanotransduction or are they broken down and recycled?\textsuperscript{30} We observed that increases in β-catenin translocation in cells cultured on POS was attenuated through inhibition of FAK turnover. This suggests that FAK turnover plays a role in β-catenin translocation and that β-catenin participates in nuclear translocation after focal adhesion binding. A study by Kam and Quaranta has also provided evidence to suggest a similar conclusion.\textsuperscript{30} By labeling β-catenin with a GFP+ fluorophore they concluded that in E-cadherins, a similar adhesion junction, disassembly allowed for the accumulation of GFP+ β-catenin in the endocytic recycling compartment.\textsuperscript{7,8,30,31} GFP+ β-catenin then participated in nuclear translocation, increasing transcriptional activity as measured by TOPFLASH reporter.\textsuperscript{7} Various other studies have suggested a similar result, although these studies evaluated β-catenin translocation from cadherin complexes as well.\textsuperscript{7,8,31} It has also been noted in previous studies that β-catenin is sequestered by both adhesions and the transcriptional pool, and therefore when decreases in adhesions occur, upregulation of β-catenin transcriptional activity can occur.\textsuperscript{15,32–33} This has led to the suggestion that adhesions function as a reservoir for β-catenin, allowing rapid increases in intracellular β-catenin concentration in response to various stimuli,\textsuperscript{34} a similar mechanism to what is proposed in this study.

While these data provide support for the proposed focal adhesion initiated, β-catenin mediate mechanism of substrate transduction further research is needed. For instance, as noted previously substrate physical properties play a significant role in directing osteogenic differentiation. The Young’s modulus of TCPS is approximately 1–3 × 10\textsuperscript{3} kPa\textsuperscript{35,36} while the Young’s modulus for PCL/HA substrates varies highly depending on PCL molecular weight and HA concentration. Bulk PCL’s Young’s modulus is approximately 3 × 10\textsuperscript{5} kPa\textsuperscript{37} while HA has a Young’s modulus of roughly 5–60 GPa depending on synthesis methodology, carbonate content, size, and structure.\textsuperscript{38} Based on literature of similar PCL/HA composites, the Young’s modulus of POS can be hypothesized to be in the 60 MPa range.\textsuperscript{39} While not the focus of this work, it is well documented in the literature that increasing substrate stiffness can induce greater commitment of cells to the osteogenic lineage, and as noted here the POS does have a greater Young’s modulus than the TCPS and is likely a contributory factor. However, studies done on osteogenic differentiation with nanoposts, where stiffness is more easily tunable, provided evidence suggesting stiffness was not singularly responsible for directing osteogenic differentiation and other mechanisms must be involved.\textsuperscript{24,25} Interestingly, the most osteogenic substrates also observed increases in focal adhesion formation regardless of substrate, stiffness, nanopost height or width, or material used.\textsuperscript{25,60} This suggests that the formation of the adhesion and the cell-substrate interaction are likely key contributors to osteogenic commitment and are why we then examined focal adhesion induced mechanisms of osteoblastic differentiation shown in this study.

FAK actively participates in numerous cellular pathways, making it difficult to determine if the observed changes are due strictly to β-catenin liberation from focal adhesion or due to changes in other proteins affected by FAK.\textsuperscript{41} For example, one downstream pathway directly affected by changes in FAK is the Ak strain transforming/mammalian mechanistic target of rapamycin (AKT/mTORC) pathway. Upon phosphorylation, FAK stimulates the phosphorylation of AKT.\textsuperscript{41,42} In addition, tension developed within the cytoskeleton during focal adhesion maturation can increase mTORC2 activity which results in the phosphorylation of AKT and an upregulation in its activity.\textsuperscript{31} Significantly, AKT is a potent inactivator of GSK-3β, which phosphorylates β-catenin in the cytoplasm and leads to its eventual degradation, providing a potential mechanism by which changes in FAK activity can regulate β-catenin activity.\textsuperscript{34} Moreover, studies have
determined that upon activation of mTORC2 through increased cell tension, significant increases in β-catenin concentration in the cytoplasm occurs. However, mTORC2 is predominately located in the endoplasmic reticulum, suggesting it may not be responsible for the rapid changes we observed in response to POS but may instead work at a distance well after adhesions have formed. Additionally, in various cancers, such as ovarian, colon, and intestinal cancer, where focal adhesions play a critical role in tumor metastasis, it has been observed that FAK directly influences β-catenin activity. It does so through phosphorylation of GSK-3β and by upregulating AKT phosphorylation and proline rich-tyrosine kinase 2 (PYK2) activity, which directly inhibit GSK-3β. Taken together, it is clear further studies that evaluate β-catenin bound directly at the focal adhesion are necessary to determine that the observed changes are not due to other contributing factors.

The mechanism by which the transduction of substrate characteristics occurs is highly complex and it is challenging to segregate the numerous effects of proteins such as β-catenin and FAK which may participate in cell transduction. The study presented here evaluated the effect of focal adhesion turnover on β-catenin localization, transcriptional activity, and osteoblastic differentiation to evaluate a proposed focal adhesion initiated, β-catenin mediated mechanism of substrate transduction. The evidence suggests that focal adhesion turnover and the observed changes in β-catenin translocation are related, however further study is needed to discern whether β-catenin translocation is localized at focal adhesions specifically or a result of other cellular pathways.

5 | CONCLUSION

This study examined how inhibition of focal adhesion turnover affects β-catenin translocation, β-catenin transcriptional activity, and the resulting osteoblastic differentiation to evaluate the proposed focal adhesion initiated, β-catenin mediated mechanism of substrate surface transduction. To evaluate if the previously observed translocation and increase in transcriptional activity was a result of focal adhesion turnover, inhibiting focal adhesion turnover was required. We found that introduction of PF-573228, an inhibitor of FAK that reduces focal turnover, inhibiting focal adhesion turnover was required. We observed that FAK directly influences β-catenin activity. It does so through phosphorylation of GSK-3β and by upregulating AKT phosphorylation and proline rich-tyrosine kinase 2 (PYK2) activity, which directly inhibit GSK-3β. Taken together, it is clear further studies that evaluate β-catenin bound directly at the focal adhesion are necessary to determine that the observed changes are not due to other contributing factors.

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The evidence provides support for the hypothesis that the observed changes in β-catenin translocation, transcriptional activity, and the resulting osteoblastic differentiation are directly related to the more rapid turnover in focal adhesions caused by the POS. Furthermore, the data presented here and previously by our group provides evidence for the support of our proposed mechanism of substrate surface transduction, in which focal adhesion turnover liberates β-catenin from the focal adhesion complex, allowing it to then translocate to the nucleus where it can participate in the transcriptional regulation of osteogenesis. However, more study is needed to determine if the β-catenin we observe translocating is specifically released from the focal adhesion complex itself or is a result of changes in other cell pathways such as the AKT/mTORC which can be regulated by FAK as well.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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