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RESEARCH ARTICLE

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The mechanotransduction of MLO-Y4 cells is disrupted by the senescence-associated secretory phenotype of neighboring cells

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Abstract

Age-related bone loss is attributed to the accumulation of senescent cells and their increasing production of inflammatory cytokines as part of the senescence-associated secretory phenotype (SASP). In otherwise healthy individuals, osteocytes play a key role in maintaining bone mass through their primary function of responding to skeletal loading. Given that osteocytes' response to loading is known to steadily decline with age, we hypothesized that the increasing presence of senescent cells and their SASP inhibit osteocytes' response to loading. To test this hypothesis, we developed two in vitro models of senescent osteocytes and osteoblasts derived from MLO-Y4 and MC3T3 cell lines, respectively. The senescent phenotype was unique to each cell type based on distinct changes in cell cycle inhibitors and SASP profile. The SASP profile of senescent osteocytes was in part dependent on nuclear factor-κB signaling and presents a new potential mechanism to target the SASP in bone. Nonsenescent MLO-Y4 cells cultured with the SASP of each senescent cell type failed to exhibit changes in gene expression as well as ERK phosphorylation and prostaglandin E2 release. The SASP of senescent osteocytes had the largest effect and neutralizing interleukin-6 (IL-6) as part of the SASP restored osteocytes' response to loading. The loss in mechanotransduction due to IL-6 was attributed to a decrease in P2X7 expression and overall sensitivity to purinergic signaling. Altogether, these findings demonstrate that the SASP of senescent cells have a negative effect on the mechanotransduction of osteocytes and that IL-6 is a key SASP component that contributes to the loss in mechanotransduction.

KEYWORDS
aging, cellular senescence, interleukin-6, mechanotransduction, osteocytes

1 | INTRODUCTION

Osteocytes are ideally placed throughout the cortex of bone to sense mechanical loading and initiate bone formation to maintain bone mass and overall strength (Bonewald & Johnson, 2008; Uda et al., 2017). However, the anabolic response to loading declines with age (Holguin et al., 2014; Rubin et al., 1992; Srinivasan et al., 2003; Turner et al., 1995), limiting the anabolic effect of physical loading and exercise in adults (Bielemann et al., 2013; Forwood & Burr, 1993; Gomez-Cabello et al., 2012; Kohrt, 2001; Marques et al., 2012; Nikander et al., 2010; Rubin et al., 1992). The lack of response to loading at the tissue level is in part attributed to a decline in

osteocyte function and shift in mechanotransduction (Galea et al., 2017; Hemmatian et al., 2017; Jilka & O'Brien, 2016; Srinivasan et al., 2012). Age-related changes in osteocytes' response to loading include a decrease in intracellular calcium signaling (Morrell et al., 2020) alongside an incapacity to regulate sclerostin and wnt ligands (Gardinier et al., 2018; Holguin et al., 2016). In vitro studies involving primary osteocytes have also found aging to shift the biological response to loading, specifically related to nitric oxide (NO) and cyclooxygenase-2 production (Chalil et al., 2015). The underlying mechanisms by which osteocytes' response to loading is lost with age remains unclear and presents a limitation in developing new therapeutic approaches toward preventing age-related bone loss.

One of the hallmarks of the aging process is the accumulation of senescent cells and their inflammatory phenotype (Baker et al., 2011; Farr et al., 2017; Roos et al., 2016). Senescent cells are characterized by extensive genomic changes that lead to proliferative arrest and the release of inflammatory cytokines as a part of the senescence-associated secretory phenotype (SASP) (Coppe et al., 2010; Freund et al., 2010; Hernandez-Segura et al., 2018). The inflammatory cytokines released by senescent cells contribute to a state of low-grade chronic inflammation that extends beyond their local environment and impact the function of neighboring tissues (Xu et al., 2018). Eliminating senescent cells or targeting their SASP in aged mice has been shown to prevent bone loss by increasing bone formation and reducing bone resorption (Farr et al., 2017). Although the shift in bone remodeling is attributed to gains in osteocyte function, the extent to which osteocyte mechanotransduction is affected by senescent cells is entirely unclear.

Senescent cells within the bone microenvironment produce several inflammatory cytokines that include interleukin-1α (IL-1α), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) (Farr et al., 2016, 2017). Independent of the SASP, cytokines can have differing effects on osteocytes' mechanotransduction (Bakker et al., 2009, 2014). Therefore, the purpose of this study was to examine how osteocytes' response to mechanical stimuli is affected by the unique cytokine profile of senescent osteocytes' and osteoblasts' SASP. Based on our results, the SASP of senescent osteocytes suppresses the mechanotransduction of normal osteocytes to a greater degree than the SASP produced by senescent osteoblasts. Furthermore, the production of IL-6 by senescent osteocytes was largely regulated by aberrant nuclear factor-κB (NF-κB) signaling and was responsible for suppressing the mechanotransduction of normal osteocytes. Altogether, these findings demonstrate that senescent osteocytes accumulating in cortical bone have a negative effect on the mechanotransduction of neighboring osteocytes through the paracrine function of IL-6 as part of the SASP.

2 | METHODS

2.1 | Reagents and antibodies

Fetal bovine serum (FBS) and calf serum (CS) were obtained from Sigma-Aldrich along with culture media, trypsin EDTA, antibiotics, and adenosine 5′-triphosphate (ATP). Antibodies against total ERK1/2 (#9107) and phosphorylated ERK1/2 (#9101) were purchased from Cell Signaling Technologies. The antibody for P2X7 (#APR-008) was purchased from Alomone Labs. Neutralizing antibodies against IL-6 (#AF-406) and IL-1α (#AF-400) were purchased from R&D Systems, Inc. Unless noted, all other chemicals were purchased from Invitrogen.

2.2 | Cell culture

Osteocyte-like MLO-Y4 cells were cultured in Petri dishes coated with rat type-1 collagen (BD Biosciences). Growth media consisted of α-minimum essential medium (α-MEM) supplemented with 2.5% FBS, 2.5% CS, and 1% penicillin–streptomycin (P/S). Osteoblast-like MC3T3-E1 cells were cultured in α-MEM containing 10% FBS and 1% P/S. Cells were maintained in a humidified incubator at 37°C and 5% CO_2 and were not allowed to exceed 70%–80% confluency to maintain the phenotype of each cell type.

2.3 | Generation of SASP conditioned media

Senescence was induced by exposing each cell type to a single dose (10 Gy) of cesium irradiation in a Cs-137 irradiator. Radiated and non-irradiated controls were then cultured for an additional 10 days before collecting conditioned media over a period of 24 h. The conditioned media was centrifuged and filtered to remove cell debris. After collecting the conditioned media, cells were fixed with 4% paraformaldehyde and stained for senescence-associated β-galactosidase (SA-βGal) activity at a pH of 6.0 using freshly prepared 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in phosphate-buffered saline (PBS). Additional samples of senescent cells were used to collect messenger RNA (mRNA) samples to examine changes in gene expression.

2.4 | In vitro fluid flow

Nonsenescent MLO-Y4 cells were seeded on rat type-1 collagen-coated glass slides at a density of 1×10^3 cells/cm^2. After 24 h, the media were replaced with conditioned media from senescent or nonsenescent cells and then cultured for another 2 days. Cells were then washed with PBS and exposed to oscillatory fluid flow (OFF) using a parallel plate chamber as described previously (Jacobs et al., 1998). Briefly, laminar fluid flow in the chamber was controlled by a crank-rocker mechanism, which generated a sinusoidal flow at 1 Hz with a peak shear stress of 20 dynes/cm^2. Flow media consisted of α-MEM with 1% FBS and 1% P/S.

2.5 | Gene expression

Trizol extracted mRNA was purified (RNeasy® Mini Kit; Qiagen) and then used to generate complementary DNA (cDNA) (Taqman cDNA Synthesis Kit; Applied Biosystems). An Applied BioSystems 7500

RealTime PCR machine was used for real-time quantitative PCR (qRT-PCR) along with the following Taqman primers: *Tbp* (Mm01277042), *Tnfsf11* (Rankl, Mm00441906), *Tnfrsf11b* (Opg, Mm00435454), *Ptgs2* (Mm00478374), *Dkk1* (Mm00438422), and *Wnt10b* (Mm00442104). All samples were normalized to their respective expression of *Tbp* using the $2^{-\Delta\Delta C_t}$ method.

2.6 | Western blot analysis

Cell lysates were prepared using radioimmunoprecipitation assay buffer (140 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% NP-40, pH 7.5) containing 1 mM of phenylmethylsulfonylfluoride (PMSF) and Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Cell lysates were separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with Intercept® Blocking Buffer (LI-COR, Inc.) and then incubated overnight at 4°C with appropriate primary antibodies followed by IRDye® 800CW secondary antibodies (1:10,000 dilution, LI-COR, Inc.) for 1 h at room temperature. Bands were visualized using the LiCor Odyssey CLx scanner and densitometry was determined using the NIH ImageJ software.

2.7 | Release of secondary messengers

ATP and prostaglandin E2 (PGE2) concentrations were measured using the luciferin–luciferase reaction (ATP Bioluminescence Assay Kit HS II; Roche) and the Prostaglandin E2 ELISA Kit (#514010; Cayman Chemical). NO concentration was measured using a Griess reagent (1% sulfanilamide, 0.1% naphthylethylene-diamine-dihydrochloride, and 2.5 M H_3PO_4). Concentrations for ATP, NO, and PGE2 were normalized by the respective cell lysate protein concentration, which was measured via Pierce™ BCA Protein Assay Kit (Thermo Scientific Scientific).

2.8 | Statistical analysis

All outcome measures are reported as the group mean ± standard deviation. For each measurement variable, a one-way analysis of variance alongside Tukey's post hoc testing to identify significant differences between groups. A p value less than 0.05 was considered significant.

3 | RESULTS

3.1 | Osteocytes and osteoblasts display distinct senescent phenotypes and SASP

Irradiation of MC3T3 and MLO-Y4 cells induced a senescent phenotype that was characterized 10 days later by an increase in SA-βGal (Figure 1a). Cells positive for SA-βGal were also noted as being larger in size compared with nonsenescent controls. Irradiated MC3T3 cells exhibited significantly increased gene expression of *p16^{ink4a}* and decreased expression of *p53*, while *p21* expression was not significantly changed compared with non-irradiated controls (Figure 1b). Irradiated MLO-Y4 cells exhibited a significant increase in *p16^{ink4a}* and *p21* expression compared with nonirradiated controls. Both cell lines expressed elevated levels of *Il-6*, *Il-1α*, *Tnf-α*, and *Cxcl15* (mouse analogous to IL-8) 10 days after irradiation.

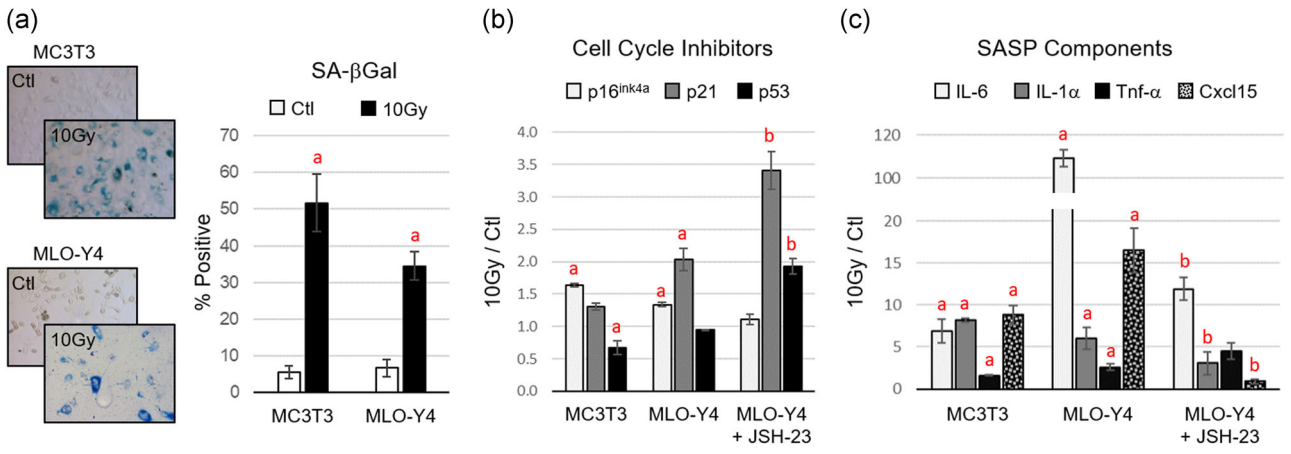

FIGURE 1 Irradiating MC3T3 and MLO-Y4 cells produce distinct senescent phenotypes and SASP. (a) SA-βGal was evident 10 days after irradiation (10 Gy). The mRNA expression of (b) cell cyclic inhibitors *p16^{ink4a}*, *p21*, and *p53* as well as (c) inflammatory cytokines common to SASP were measured and normalized against *Tbp* plotted as a fold change compared with NM. Significant differences with a p value <0.05 are denoted by "a" for comparisons to respective nonsenescent control and "b" for comparisons to the respective senescent MLO-Y4 control (mean ± standard deviation, $n = 4$). IL-6, interleukin-6; SA-βGal, senescence-associated β-galactosidase; SASP, senescence-associated secretory phenotype; TNF-α, tumor necrosis factor-α

To examine the role of NF-κB in regulating senescent cells' production of cytokines, the senescent MLO-Y4 cells were treated for 2 days with JSH-23 to inhibit the transcriptional activity of NF-κB. Treatment of senescent cells with JSH-23 significantly increased p21 further and p53 when compared with the expression before treatment (Figure 1b). In addition, JSH-23 treatment decreased the expression of *Il-6*, *Il-1α*, and *Cxcl15* when compared with the expression before treatment (Figure 1c). Overall, JSH-23 had the greatest effect on *Il-6* and *Cxcl15*.

3.2 | OFF-induced gene expression is impaired by the SASP

Nonsenescent osteocytes were cultured with conditioned media from senescent osteoblasts (OB-SASP) and senescent osteocytes (Ocy-SASP) for 2 days before static or OFF conditions (Figure 2a). Under static conditions, both OB-SASP and Ocy-SASP significantly increased *Rankl* expression by three- and sevenfold, respectively, while only Ocy-SASP produced a significant decrease in *Opg* and *Dkk1* by 53% and 80%, respectively, when compared with static controls cultured with conditioned media from non-senesenct cells (NM) (Figure 2b).

For osteocytes cultured with NM, OFF significantly decreased *Rankl* and *Dkk1* expression by 55% and 48%, respectively, and significantly increased *Ptgs2* and *Wnt10b* expression by ~2- and ~2.5-fold, respectively, when compared with static NM controls. For osteocytes cultured with OB-SASP and Ocy-SASP, OFF had no significant effect on *Rankl*, *Opg*, *Ptgs2*, or *Dkk1* expression when compared with their respective static controls, while OFF significantly increased *Wnt10b* expression only in osteocytes treated with OB-SASP.

3.3 | Changes in osteocyte mechanotransduction are mediated by IL-6 more than IL-1α

For osteocytes cultured with NM, OFF significantly increased NO and PGE2 release by 2.5- and 8-fold, respectively, when compared with static controls (Figure 3a,b). The release of NO and PGE2 in response to OFF significantly decreased for osteocytes cultured with Ocy-SASP when compared with NM controls exposed to the same OFF. However, OFF still induced a small but significant increase in PGE2 release when compared with static Ocy-SASP controls. For osteocytes cultured with OB-SASP, the release of NO and PGE2 was not statistically different compared to either OB-SASP static controls or NM controls subjected to the same OFF. Both OB-SASP and Ocy-SASP inhibited ERK phosphorylation induced by OFF (Figure 3c).

To understand which component of the Ocy-SASP caused the loss in mechanotransduction, IL-6 and IL-1α were neutralized by adding antibodies while culturing nonsenescent MLO-Y4 cells with Ocy-SASP. Neutralizing IL-1α in the Ocy-SASP failed to restore osteocytes' response to OFF while neutralizing IL-6 restored osteocytes' release of NO and PGE2 in response to OFF alongside ERK phosphorylation (Figure 4). In particular, PGE2 release in response to OFF was significantly greater in osteocytes treated with Ocy-SASP and the IL-6 antibody when compared

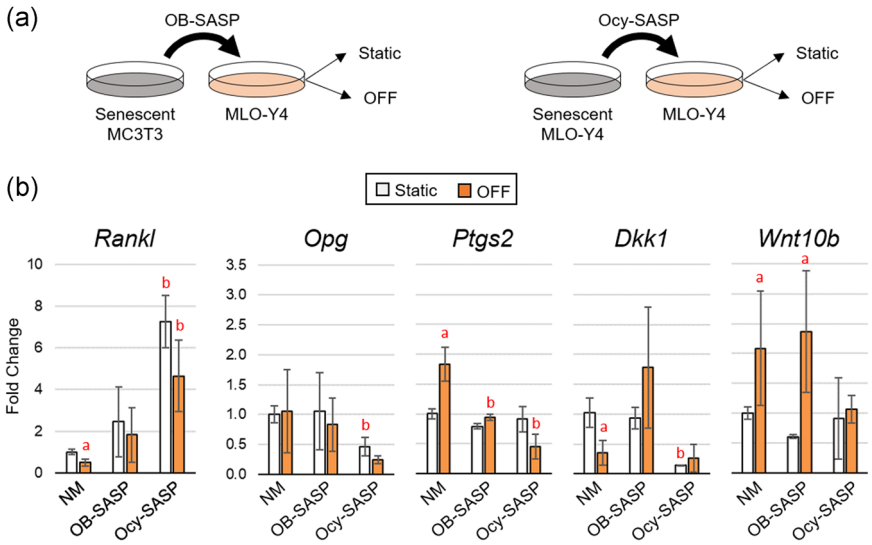

FIGURE 2 Fluid flow-induced gene expression of nonsenescent MLO-Y4 cells is inhibited by the SASP of senescent osteocytes and osteoblasts. (a) Our study design cultured nonsenescent MLO-Y4 cells with conditioned media from senescent osteoblasts (OB-SASP) or osteocytes (Ocy-SASP) for 2 days and then subjected them to 2 h of static or OFF conditions. (b) The mRNA expression of *Rankl*, *Opg*, *Ptgs2*, *Dkk1*, and *Wnt10b* were measured relative to *Tbp* via qRT-PCR analysis and then normalized to NM static controls. Significant differences with a p value <0.05 are denoted by "a" for comparisons to respective static control and "b" for comparisons to the respective NM control (mean ± standard deviation, $n = 3$). IL-6, interleukin-6; mRNA, messenger RNA; OFF, oscillatory fluid flow; qRT-PCR, real-time quantitative PCR; SASP, senescence-associated secretory phenotype

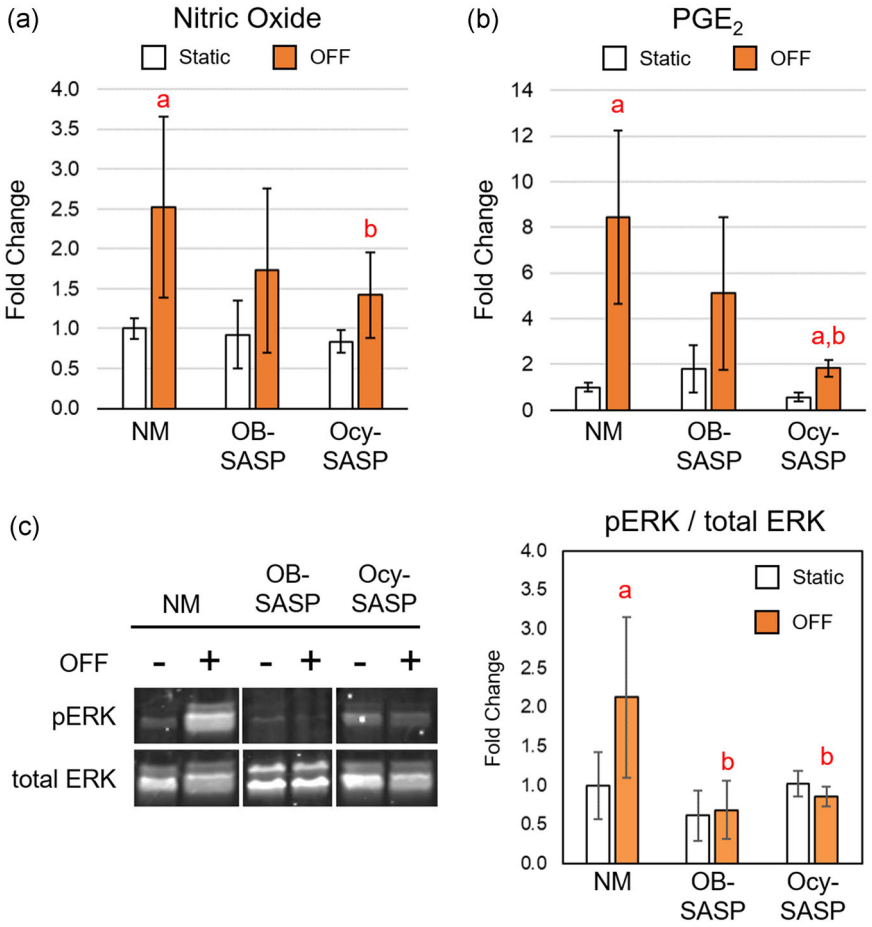

FIGURE 3 Fluid flow-induced release of secondary messengers and phosphorylation of ERK of nonsenescent MLO-Y4 cells are inhibited by the SASP of senescent osteocytes and osteoblasts. After 1 h of OFF, the extracellular concentration of NO (a) and PGE2 (b) were measured alongside intracellular phosphorylation of ERK (c). Significant differences with a p value <0.05 are denoted by "a" for comparisons to respective static control and "b" for comparisons to the respective NM control (mean ± standard deviation, $n = 4$). NO, nitric oxide; OB-SASP, senescent osteoblasts; Ocy-SASP, osteocytes; OFF, oscillatory fluid flow; PGE2, prostaglandin E2; SASP, senescence-associated secretory phenotype

with osteocytes treated with Ocy-SASP alone (Figure 4b) or osteocytes cultured with NM and the IL-6 antibody ($p < 0.001$; see Figure S1). Neutralizing IL-6 in NM did not have a significant effect on either ERK phosphorylation ($p < 0.56$) or PGE2 release ($p < 0.85$) in response to OFF (Figure S1).

3.4 | The SASP decreases osteocytes' sensitivity to purinergic signaling through P2X7

Considering PGE2 and ERK phosphorylation under loading are mediated via purinergic signaling (Li et al., 2005), we measured ATP release and found fluid flow still induced a significant increase despite Ocy-SASP (Figure 5a). However, directly treating osteocytes with ATP cultured with Ocy-SASP failed to induce ERK phosphorylation (Figure 5b). Neutralizing IL-6 in Ocy-SASP restored osteocytes phosphorylation of ERK phosphorylation in response to ATP. We then measured the expression of P2X7 given its role in regulating both ERK phosphorylation and PGE2 release under loading (Grol et al., 2009). The mRNA expression of P2X7 was not affected by the SASP of senescent cells (Figure 5c). However, protein levels of P2X7 were significantly attenuated by 90% and then restored by 50% when neutralizing IL-6 (Figure 5d).

4 | DISCUSSION

Our findings demonstrate that the mechanotransduction of healthy osteocytes is negatively affected by the SASP of senescent osteocytes and osteoblasts. The senescent phenotype of osteocytes and osteoblasts were distinctly different between cell types based on their unique changes in cell cycle inhibitors p21 and p53. The production of IL-6 by senescent osteocytes was in part dependent on NF-κB signaling and the fact that inhibiting NF-κB was able to significantly reduce IL-6 expression presents a new potential mechanism to targeting the SASP in bone. By comparison, senescent osteocytes have a larger effect on the mechanotransduction of healthy osteocytes. A similar loss in PGE2 release and *Wnt10b* expression in response to loading is reported to occur across different models of aging (Galea et al., 2017; Klein-Nulend et al., 2002). Of the various SASP components, IL-6 had the largest contribution to the loss in mechanotransduction. Lastly, senescent osteocytes negatively impacted the mechanotransduction of healthy osteocytes by way of decreasing the sensitivity to purinergic signaling, most notably through the loss of P2X7 expression. The absence of P2X7 in knockout mice is detrimental to the anabolic response to loading (Li et al., 2005). In aged mice, the extent to which P2X7 function remains unclear and warrants further examination based on our findings.

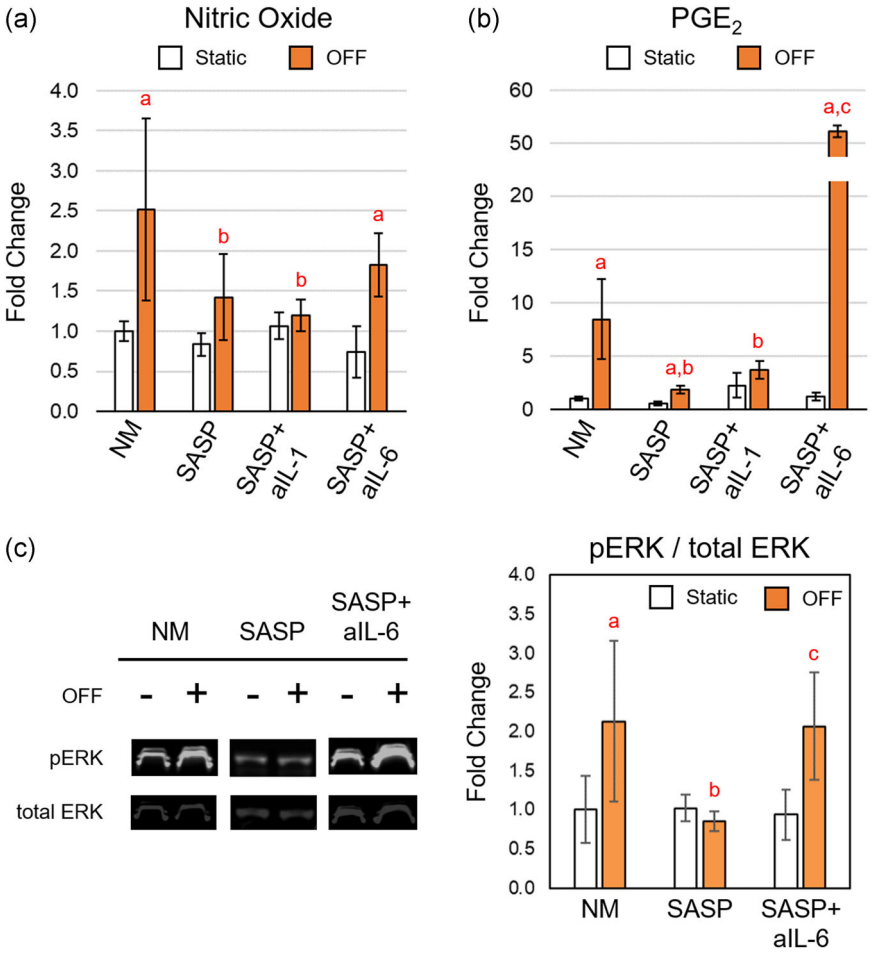

FIGURE 4 Neutralizing IL-6 of the SASP restores osteocytes' anabolic response to fluid flow. Conditioned media from senescent MLO-Y4 cells (SASP) was supplemented with neutralizing antibodies for IL-1 (aIL-1) or IL-6 (aIL-6). MLO-Y4 cells were cultured for 2 days with conditioned media from senescent osteocytes (SASP) with and without the neutralizing antibodies aIL-1 or aIL-6. After 1 h of OFF, the extracellular concentration of NO (a) and PGE2 (b) were measured alongside intracellular phosphorylation of ERK (c). Significant differences with a p value <0.05 are denoted by "a" for comparisons to respective static control and "b" for comparisons to the respective NM control, and "c" for comparisons to respective SASP control (mean ± standard deviation, $n = 4$). IL-6, interleukin-6; NO, nitric oxide; OFF, oscillatory fluid flow; PGE2, prostaglandin E2; SASP, senescence-associated secretory phenotype

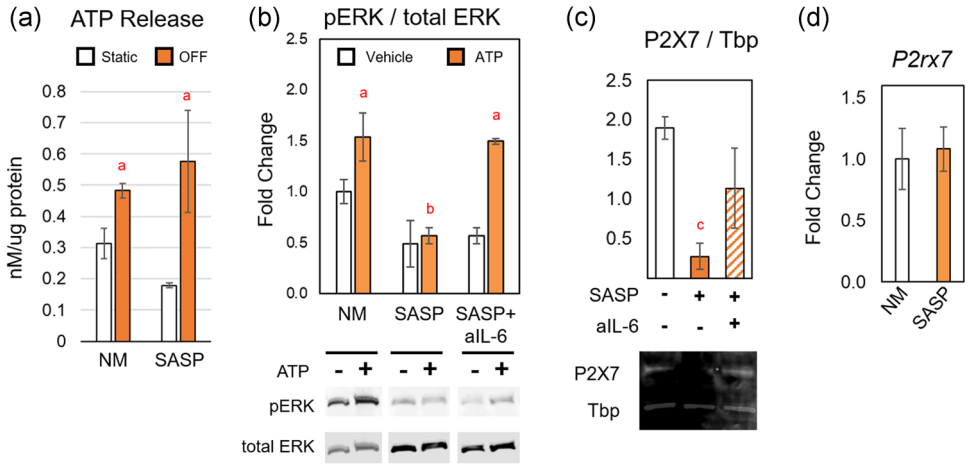

FIGURE 5 Osteocytes' functional response to ATP is inhibited by the SASP of senescent osteocytes through IL-6. (a) Extracellular ATP was measured in response to OFF in MLO-Y4 cells following 2 days of culture with conditioned media from senescent osteocytes (SASP) or nonsenescent osteocytes (NM) (mean ± standard deviation, $n = 3$). (b) Phosphorylation of ERK was measured via western blot and quantified for MLO-Y4 cells treated with ATP (100 μM for 15 min). Before treatment, MLO-Y4 cells were cultured with or without SASP as well as the neutralizing antibody for IL-6 (aIL-6). (c) Western blot and quantified expression of P2X7 receptor relative to Tbp in MLO-Y4 cells treated with or without SASP as well as aIL-6. (d) The mRNA expression of *P2rx7* was measured relative to Tbp and normalized to NM controls. Significant differences with a p value <0.05 are denoted by "a" for comparisons to respective vehicle-treated control and "b" for comparisons to the respective ATP-treated NM control and "c" for comparisons to respective SASP control (mean ± standard deviation, $n = 4$). ATP, adenosine 5′-triphosphate; IL-6, interleukin-6; mRNA, messenger RNA; NO, nitric oxide; OFF, oscillatory fluid flow; SASP, senescence-associated secretory phenotype

Both models of senescent cells showed an increase in *p16*^{ink4a} alongside decreased proliferation, increased cell size, and increased β-galactosidase activity. The increase in *p16*^{ink4a} in particular is consistent with the age-related changes observed in mice (Farr et al., 2017). However, the decrease in *p53* expression among irradiated MC3T3 cells was unexpected given the prominent function of p53 in regulating cell cycle arrest. The lack of p53 expression suggests that p16^{ink4a} signaling through retinoblastoma may play a larger role in cell cycle arrest through the classical p53 pathway (Kumari & Jat, 2021). At the same time, the senescent phenotype of irradiated MLO-Y4 cells did not include elevated p53 expression, but increased p16^{ink4a} and p21. In contrast, oxidative stress is reported to produce a senescent phenotype marked by elevated p21 and p53 without changes in p16^{ink4a} (Wei et al., 2021), suggesting that the onset of senescence in these cells is dependent on the type of trigger.

Similar to other studies, the SASP profile of the senescent MLO-Y4 cells was in part regulated by NF-κB. In particular, NF-κB contributed to the expression of IL-6, IL-1α, and Cxcl15, but not TNF-α. In contrast, inhibition of NF-κB increased p53 and p21 expression, suggesting that cell-cycle arrest in the senescent MLO-Y4 cells is maintained independent of NF-κB. Although cross-talk between NF-κB and p53 can contribute to the senescent phenotype, they are not necessarily mutually inclusive. In fact, RNAi studies have found p53 and p16^{ink4a} expression to remain unaffected when suppressing NF-κB in senescent cells despite significant reductions in the inflammatory profile (Chien et al., 2011; Salminen et al., 2012). In addition, studies have also found p53 and NF-κB to counteract each other and that their increased activity with senescence is regulated independent of each other (Ak & Levine, 2010; Salminen & Kaarniranta, 2011). Overall, NF-κB appears to play a larger role in regulating the SASP and based on our findings may provide a novel target toward reducing the paracrine function of senescent cells.

The SASP profile of senescent osteocytes includes numerous cytokines and chemokines. Of those examined in this study, we found the relative increase in IL-6 expression to be greater than that of IL-1α, TNF-α, or Cxcl15. Based on our findings, IL-6 played a significant role in inhibiting the mechanotransduction of osteocytes. Neutralizing IL-6 when treating osteocytes with the SASP restored ERK phosphorylation alongside the release of PGE2. Of note, we found the release of PGE2 exceeded that of controls when neutralizing IL-6, suggesting that the other SASP components may enable osteocytes to compensate against the effects of IL-6. Independent of the SASP, directly treating osteocytes with IL-6 has little effect on the release of NO, while treating with IL-1α or TNF-α can completely abolish NO release alongside the release of PGE2 (Bakker et al., 2009, 2014) As a result, it was surprising that neutralizing the SASP component IL-1α did not restore osteocytes' release of NO or PGE2. The discrepancy between these studies may extend from differences in concentrations. Unfortunately, the concentration of IL-1α was not quantified in our study and presented a limitation to understanding the relative production of each SASP component at the protein level. However, these findings demonstrate the unique function of IL-6 as part of the SASP and the inhibitor effect on osteocyte mechanotransduction.

The release of PGE in response to fluid flow is mediated by purinergic signaling (Genetos et al., 2007; Li et al., 2005), and is an essential component to the induction of bone formation (Galea et al., 2011; Jee & Ma, 1997). Given that ATP release was still evident despite exposure to the SASP, the lack of PGE2 signaling was then attributed to a lack of response to ATP, namely, through the P2X7 receptor. Decreased P2X7 expression was only observed at the protein level, suggesting a direct or indirect interaction between the IL-6 receptor and P2X7. Considering that activation of the IL-6 receptor induces endocytosis through caveolin phosphorylation (Cendrowski et al., 2016) and that phosphorylation of caveolin also causes endocytosis of P2X7 (Barth et al., 2007; Gangadharan et al., 2015), it is possible that exposure to IL-6 facilitates the endocytosis and degradation of P2X7 independent of changes in gene expression. Overall, the underlying mechanisms by which IL-6 as part of the SASP causes a loss in P2X7 receptor remains unclear, but warrants further examination in attempts to understand how to maintain osteocytes' sensitivity to purinergic signaling.

Our findings also suggest that the SASP of senescent osteocytes has a larger impact on bone metabolism than senescent osteoblasts. This is consistent with previous work in which eliminating senescent cells, including senescent osteocytes, effectively prevents bone loss (Farr et al., 2017), while other models that do not eliminate senescent osteocytes exhibit no changes in bone loss (Kim et al., 2019). Although the exact mechanisms by which bone formation was restored is still unclear, our findings suggest potential gains in mechanotransduction may be a contributing factor. In particular, osteocytes' gains in Wnt10b expression under loading would increase osteoblast recruitment and activation (Bennett et al., 2005; Stevens et al., 2010). Gains in PGE2 signaling are also expected to restore osteocytes capacity to downregulate *Sost* under loading (Galea et al., 2011). Unfortunately, the use of MLO-Y4 cells did not allow us to measure changes in *Sost* expression. Despite this limitation, our findings add to the emerging role of senescent osteocytes and their negative effect on bone mass and mechanotransduction. Another limitation in this study was the lack of other senescent cell types, such as fibroblasts or adipocytes, which also express high levels of IL-6 as part of their SASP alongside several others specific to their phenotype (Farr et al., 2016). Given the paracrine function of the SASP, other tissues or cell types may also effect osteocytes' mechanotransduction and would be worth exploring further in future studies.

In conclusion, our study demonstrates that the mechanotransduction of otherwise healthy osteocytes is inhibited by the SASP of neighboring senescent osteocytes. Furthermore, IL-6 appears to be a key component of the SASP contributing to the loss in mechanotransduction by decreasing osteocytes' sensitivity to purinergic signaling. Altogether, our findings suggest that the accumulation of senescent osteocytes may contribute to age-related bone loss through the loss of mechanotransduction and that targeting SASP component IL-6 has the potential to enhance the anabolic response to loading.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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