Low Senescent Burdens Rewire Osteocyte-ECM Micromechanics in 3D Co-Culture

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INTRODUCTION: Aging bone accumulates senescent osteocytes whose secretome perturbs perilacunar remodeling and mechanotransduction. Although canonical senescence markers (e.g., p16, p53/p21, SA-β-gal) are widely used, they vary by context and do not report the mechanical consequences of senescence on the osteocyte niche. *Here we pose a controlled question:* ***do small, physiologic burdens of senescent osteocytes (e.g., 5% versus 15%) measurably reprogram cell stiffness and ECM micromechanics in 3D?*** To test this, we introduce a tissue-mimetic 3D co-culture of healthy and senescent osteocytes and quantify cell-ECM micromechanical signatures as a functional readout of senescent burden (**Fig. 1**). Defining such biophysical markers addresses a key gap: *linking senescent cell accumulation to niche-level mechanical dysfunction that underlies age-related skeletal fragility.*

METHODS: Senescence was induced in immortalized osteocytes (MLO-Y4) by a 1 Gy irradiation dose (10-min exposure), followed by 28 days of culture with media changes every 4 days (Fig. 1A). Parallel plates of non-irradiated cells served as healthy controls. On day 28, co-cultures were prepared at defined senescent:healthy ratios of 0:100, 5:95, 10:90, 15:85, and 100:0 (**Fig. 1A**). Cell mixtures were encapsulated in a bioink of 5% (v/v) PEGDA, 5% (w/v) GelMA, and 0.15 mg/mL rat-tail type I collagen, and 3D-printed into scaffolds (**Fig. 1B-D**). Constructs were maintained for 7 or 14 days before fixation in 4% paraformaldehyde (PFA) and subsequent cryosectioning. Micromechanical properties of both scaffold (ECM region) and cells were mapped via nanoindentation (Pavone, Optics11Life; fiber-optic interferometry nanoindenter) using a 3 μm radius and 0.51 N/m stiffness probe. Indentation data were analyzed with Hertzian contact mechanics. For each group, >50 indentations were performed within the scaffold region and >100 on cells. To assess DNA damage, a TUNEL assay (indicator of DNA fragmentation) was performed, acquiring fluorescence images from 9 spatially distinct fields per group. *Mmp12* expression was evaluated by immunofluorescence, also sampling 9 fields per group. Statistical comparisons used two-way ANOVA with significance set to $p < 0.05$.

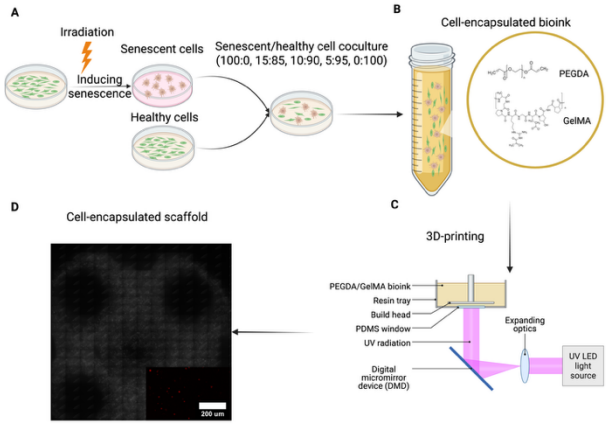

Fig. 1: *The fabrication process of the scaffold an immunofluorescent image of the osteocytes encapsulated in the ECM.*

RESULTS: At day 14, the healthy-only (0% SnC) constructs exhibited the lowest ECM effective Young's modulus among all groups (**Fig. 2A**), coincident with the highest *Mmp12* signal in that group (**Fig. 2C**), consistent with time-dependent matrix remodeling/softening. By contrast, on day 7, the 0% SnC ECM was relatively stiffer and displayed low *Mmp12* (**Fig. 2B-C**). Across day 7, increasing senescent burden produced an inverse coupling between compartments: cell modulus increased with SnC fraction while ECM modulus decreased (**Fig. 2A-B**). On day 14, ECM mechanics in the co-cultures (5%, 10%, 15% SnC) remained higher than healthy controls, with a modest downward trend as senescent fraction increased (5% > 10% > 15%), indicating that even low senescent burdens measurably reprogram the ECM surrounding osteocytes (**Fig. 2A**). As expected, 100% SnC constructs showed the highest TUNEL signal at both timepoints (**Fig. 2D**). In contrast, 5-15% SnC co-cultures did not differ significantly from healthy controls in TUNEL, indicating that DNA-break signal is insensitive to small senescent loads relative to the mechanical readout. A baseline TUNEL signal was detectable across all groups, as anticipated for long-term culture and processing, but did not confound the dose-responsive micromechanical signatures that discriminate 5% vs 15% senescent burden.

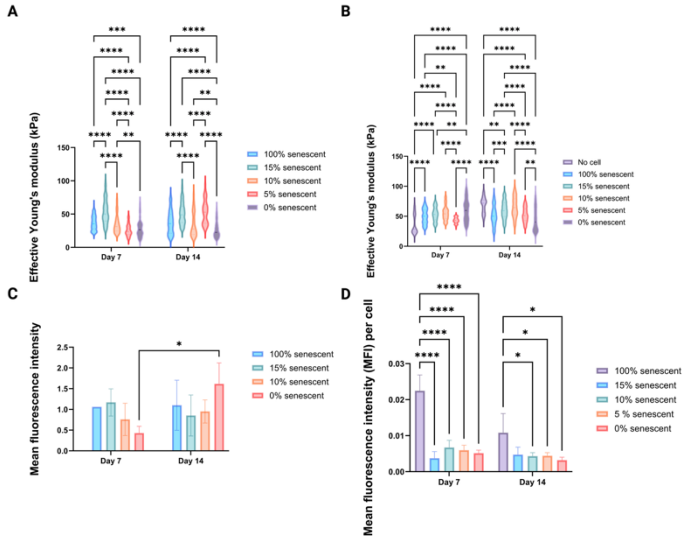

Fig. 2: *The osteocyte and ECM properties. Cell (A) and matrix (B) indentation. Mmp12 (C) and TUNEL (D) intensity.*

DISCUSSION: This study defines how small, controlled burdens of senescent osteocytes reshape both cell and ECM micromechanics in 3D. By day 7, increasing SnC fraction produced an inverse coupling across compartments (i.e., cell modulus increased while ECM modulus decreased), suggesting early, SnC-driven alterations in matrix processing (**Fig. 2B**). By day 14, the healthy-only group showed the lowest ECM modulus with highest *Mmp12*, consistent with time-dependent ECM remodeling/softening, whereas co-cultures (5-15% SnCs) remained mechanically higher than healthy controls (**Fig. 2A-C**). ***Altogether, these data support a kinetic model in which SnCs alter ECM remodeling trajectories, either by dampening protease-mediated softening, shifting the balance toward inhibitor/crosslinking programs, or slowing turnover, yielding a stiffer ECM relative to healthy controls at later time points.*** Notably, TUNEL distinguished only the extreme case (100% SnCs) and did not separate 5% vs 15% burdens, whereas the cell-ECM mechanical readouts did, indicating greater sensitivity of micromechanics to modest senescent loads.

SIGNIFICANCE: We establish micromechanical signatures as quantitative readouts of osteocyte senescent burden in a controlled 3D system. Because standard molecular markers can be variable, these ECM and cell stiffness metrics offer a functional, sensitive endpoint that discriminates 5% vs 15% SnCs. *Practically, such readouts can (i) guide matrix-centric interventions aimed at restoring osteocyte mechanosensation, (ii) serve as screening endpoints for senolytics or remodeling-targeted therapies, and (iii) inform design rules for implants and scaffolds in aging bone where preserving a healthy osteocyte-ECM mechanical niche may mitigate fragility and impaired remodeling.*