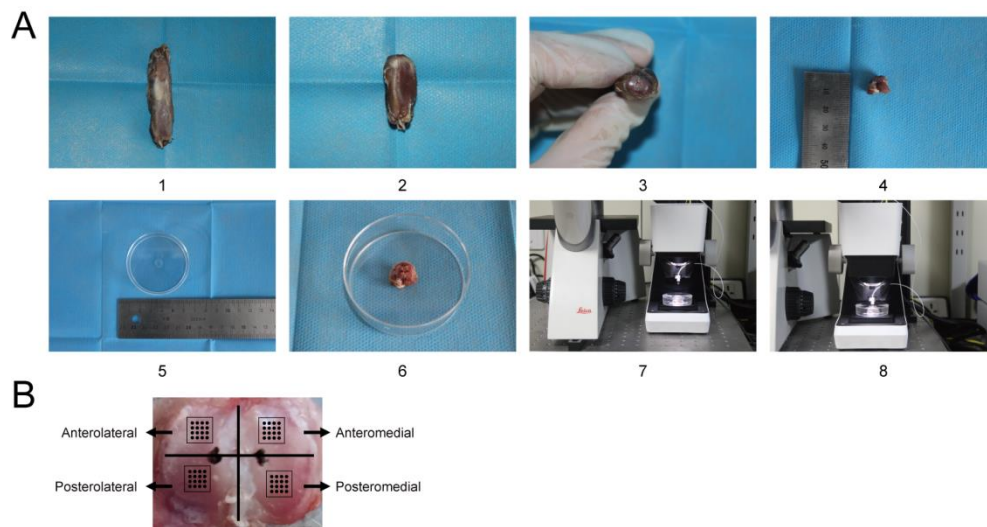
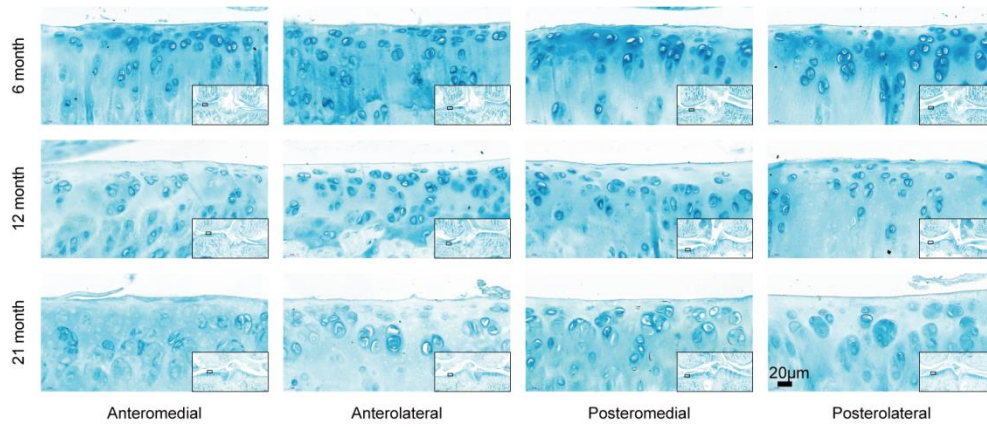


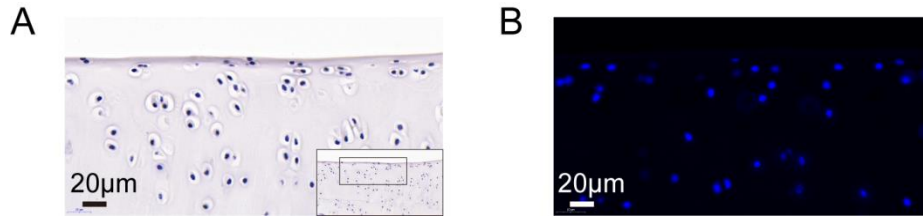
Supplementary Fig. 1 Schematic illustration of subchondral VOI selection.



Supplementary Fig. 2 Bio-nanoindentation workflow schematics. (A) Sample preparation protocol. (B) Matrix indentation mapping configuration.



Supplementary Fig. 3 Glycosaminoglycan distribution patterns visualized by Alcian blue staining across tibial plateau regions. Scale bar: 20 µm.



Supplementary Fig. 4 Representative images of negative control samples. (A) Immunohistochemical negative control. Scale bar: 20 μm. (B) Immunofluorescence negative control. Scale bar: 20 μm.

Reporting Checklist

Section/Topic	Item No.	Checklist item	Reported on page No.
Title			
Issues/topics	1	Age-Related Degeneration Initiates at the Surface: Preferential Biomechanical and Biological Decline in Rat Articular Cartilage	P1
Abstract			
Structured summary	2	Abstract: Age-related degeneration of articular cartilage is a key pathological hallmark of primary osteoarthritis (OA). As the cartilage's barrier layer, the superficial zone maintains tissue homeostasis and is increasingly recognized as the initial site of age-related cartilage degeneration. Disruption of this zone exposes deeper layers to hostile microenvironments, accelerating pan-cartilaginous changes; however, its age-related progression remains incompletely defined. We systematically characterized aging-associated alterations in the rat tibial plateau superficial zone across imaging, biomechanical, and biological parameters. Using integrated radiology, nanomechanical testing, and histomorphometry, we found that radiographic parameters did not differ significantly between 6 and 12 months. In contrast, the cartilage indentation modulus declined with age, and interregional disparities narrowed as degeneration progressed. Histology showed reduced cell density, hypertrophic chondrocytes, decreased proteoglycan content, and collagen structural disorganization in the superficial zone. Molecular profiling demonstrated downregulation of COL-II and PRG4, alongside upregulation of Caspase3, MMP13, and COL-X. These findings indicate that the anteromedial tibial plateau exhibits the earliest and most pronounced age-dependent degeneration within the superficial zone, characterized by architectural, biological, and mechanical alterations that precede significant subchondral bone remodeling. This work delineates the spatiotemporal progression of superficial zone aging, providing insights for early OA diagnosis and intervention. Keywords: Articular Cartilage; Superficial Zone; Age-Related Alterations; Biology; Biomechanics	P2
Introduction/ Background			
Justification/ Rationale/	3	Osteoarthritis (OA) is a degenerative joint disorder that	P3

Explanation		predominantly affects middle-aged and older adults, and its global prevalence rises with population aging, imposing substantial socioeconomic burdens. Articular cartilage is stratified into superficial, transitional, radial, and calcified zones. The superficial zone contains parallel-aligned collagen fibrils and 2–3 layers of flattened chondrocytes, helping maintain low friction during articulation, limit macromolecular infiltration, and distribute mechanical loads. Its topological position, composition, and functions position the superficial zone as a principal barrier against OA initiation. Accumulating evidence indicates that age-related degeneration begins in this compartment. Structural compromise of this interface allows inflammatory microenvironmental factors to access intermediate and deep strata, accelerating pan-cartilaginous deterioration. Although progenitor cells with directional differentiation and migratory repair potential reside in this zone, their capacity is insufficient for complete repair. Thus, preserving superficial-zone integrity is a pivotal objective for early OA diagnosis and intervention.	
Objectives	4	This study delineates age-associated alterations in biological properties and mechanical competence within distinct load-bearing regions of the rat tibial plateau superficial zone, with emphasis on zone-specific proteoglycan 4 (PRG4) expression dynamics. By examining OA pathogenesis through the lens of superficial-zone degeneration, this work provides conceptual and experimental grounds for early diagnosis and treatment strategies in primary OA.	P4
Methods			
Imaging	5	This study utilized two complementary imaging modalities to assess structural changes in the rat knee joint. Digital radiographs in anteroposterior and lateral projections were acquired using a small-animal X-ray system (Ultra Focus100X, Faxitron) to evaluate overall joint morphology. Subsequently, excised joints were scanned with high-resolution micro-CT (vivaCT80, SCANCO Medical AG; 70 kV, 15.6 μm slice thickness). The tibial plateau was segmented into four distinct regions. Within each region, a cylindrical volume of interest was placed in the subchondral bone for quantitative analysis of standard microstructure parameters, including bone volume fraction, trabecular number, thickness, separation, and bone-specific	P13

		surface area.	
Nanomechanical assessment	6	Nanomechanical properties of articular cartilage were assessed using a bio-nanoindenter (Piuma, Optics11). Fresh tibial specimens were mounted on hydrated substrates, and following calibration with spherical probes, automated indentation tests were performed at standardized locations across four regions using a constant displacement rate. The indentation modulus was derived from force–depth curves via Hertzian contact modeling, with multiple measurements per region averaged for statistical analysis.	P13-14
Histology	7	Histological analysis was conducted on paraffin-embedded 5 µm coronal knee sections. Following H&E staining, morphometric parameters including cartilage thickness, superficial zone dimensions, cell density, and chondrocyte hypertrophy were quantified across four tibial plateau regions using ImageJ. Proteoglycan content was assessed via Safranin O-Fast Green and Alcian Blue staining, with degeneration graded using the OARSI system. Collagen network structure was evaluated using Sirius Red staining under bright-field and polarized light microscopy.	P14
Immunohistochemistry and immunofluorescence	8	Immunohistochemical and immunofluorescence staining were performed to evaluate alterations in key functional markers within the cartilage superficial zone. Following standard protocols including antigen retrieval and serum blocking, sections were incubated with primary antibodies against COL-II, PRG4, Caspase3, MMP13, and COL-X (dilution 1:100). Immunohistochemistry employed biotinylated secondary antibodies with DAB development, while immunofluorescence used fluorophore-conjugated secondaries with DAPI counterstain. Whole-slide images were quantitatively analyzed for average optical density, mean fluorescence intensity, and positive cell rate.	P14-15
Results			
Tabulation and summarizing findings	9	1. Absence of Significant Radiographic Alterations in Rat Knee Joints with Advancing Age 2. Age-Associated Decline in Mechanical Properties of Articular Cartilage Across Distinct Weight-Bearing Regions 3. Temporal Progression of Superficial Zone Degeneration:	P4-6

		Reduced Cellularity, Chondrocytic Hypertrophy, and Proteoglycan Depletion 4. Age-Dependent Collagen Composition Shifts and Diminished PRG4 Expression in the Superficial Zone 5. Profound Alterations in Chondrocyte Biological Functions During Aging	
Result gallery	10	Figure 1-8	P22-29
Discussion			
Summary of findings	11	Utilizing an aging rat model, the present investigation focuses on the incipient pathological event of cartilage degeneration, namely, deterioration of the superficial zone. It systematically demonstrates that with advancing age, cartilage exhibits a marked decline in indentation modulus while radiographic assessments of the knee joint reveal no overt alterations. Concurrently, the extracellular matrix undergoes proteoglycan depletion alongside modifications in collagen content and architecture. The superficial zone manifests reduced chondrocyte density accompanied by cellular enlargement, elevated levels of apoptosis and hypertrophy, and a pronounced downregulation of the zone-specific protein PRG4.	P6-7
Limitations	12	First, inherent differences exist between the spontaneous rat degeneration model employed and human OA, notably due to distinct mechanical load distributions in the knee joint resulting from rodent gait kinematics. However, this model facilitates controlled, short-term investigations into OA pathological progression, an advantage not readily attainable in human studies. Second, the exclusive use of male rats means the potential influence of sex differences on the outcomes was not examined. Consequently, extrapolation of these findings to female animals and human applications requires caution. Future research should incorporate female animal models to systematically evaluate the impact of sex on the mechanisms of cartilage degeneration, thereby providing a more comprehensive understanding.	P12
Conclusion(s)	13	This multifaceted study delineates characteristic spatiotemporal patterns of age-associated degeneration within the articular cartilage superficial zone. Results demonstrate disproportionately advanced deterioration in the anteromedial tibial plateau compared to other anatomical subsectors. Biological perturbations in the superficial zone and the degradation of cartilage's mechanical properties demonstrably precede detectable subchondral bony adaptations. Principal manifestations	P12

		include progressive hypocellularity with concomitant chondrocytic hypertrophy and cluster formation; enhanced apoptosis, inflammation, and hypertrophic differentiation; and substantial PRG4 downregulation. Concurrently, collagen fibril composition exhibits structural disorganization, while proteoglycan depletion coincides with fibrotic transformation. These findings advance understanding of the pathophysiological mechanisms underpinning cartilaginous senescence and early OA pathogenesis, providing theoretical foundations for diagnostic and therapeutic modalities targeting the superficial zone.	