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EDITORIAL

Insights into osteoarthritis development from single-cell RNA sequencing of subchondral bone

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Osteoarthritis (OA) is the most common degenerative joint disease, manifested by joint pain, swelling and deformity. Meanwhile, OA is also a leading cause of disability, particularly among the elderly: an estimated 10%–15% of all adults aged over 60 years suffer from a certain degree of OA.[1 2] Obesity, joint misalignment and joint injury are key risk factors for OA.[3] It is estimated that at present 300 million people worldwide are affected by OA,[4] and the number is expected to increase by about 50% in the next decade, as a result of the ageing of the global population as well as increasing obesity and joint injuries.[5] Current treatments of OA cause very high costs of healthcare, but they are not able to effectively arrest or even slow down the degeneration process of joints.[1] A more comprehensive understanding of the pathologic cellular and molecular mechanisms that drive OA development is still urgently needed for developing effective OA therapies.

Already a decade ago, Lories and Luyten referred to OA as 'a disease of the whole joint'[6] because the pathologic changes in OA joints include articular cartilage (AC) degradation, subchondral bone (SB) thickening (sclerosis), osteophyte formation, ligament and meniscus degeneration, synovial inflammation and the abnormality of other supporting tissue surrounding the joint capsule.[7] Although AC degradation has long been considered the main cause of OA, accumulating evidence suggests that the structural alterations of SB also play an important role in the development and progression of OA. SB lies beneath the calcified cartilage and remains connected to it through a collagen type I–type II interface. The architecture of SB varies by physiological regions, from the more compact layer adjacent to the calcified cartilage (SB plate) to the subchondral trabecular bone close to the medullary cavity.[8] During physical movement, SB attenuates forces penetrated through cartilage, with the compact SB plate providing firm support and the compliance of SB trabecular module providing elasticity for shock absorption.[8] SB abnormality is highly correlated with cartilage degeneration in both animals studied in OA models and humans with OA.[9] Consistently, the improvement of the SB quality resulted in reduced AC degeneration, strongly suggesting that a normal SB structure is essential for maintaining the homeostasis of AC.[6 10] Moreover, a recent study demonstrated that, during joint movement, SB reacts with counterforce to AC and through transforming growth factor beta (TGFβ) release, which plays an important role in regulating AC homeostasis and thus OA development.[11] As AC and SB are directly attached to each other, the pathogenesis of AC also has significant impact on SB. During OA development, AC damages eventually extend into the SB layer and are regarded osteochondral defects. The defects in SB often trigger self-repair attempts and are refilled with fibrous tissues (SB sclerosis) that lack appropriate functional properties and are more susceptible to free radicals, metalloproteinases and other catabolic factors,[12–14] and, thus, can further deteriorate the SB structure. Severe cartilage loss may even lead to bone attrition—a specific type of SB loss that affects the shape of the bone by flattening or depression of the articular surface.[15] SB attrition often results in the occurrence of bone marrow lesions, which is also associated with the progression of OA.[16]

Growing evidence suggests that during OA development, a remarkable SB remodelling takes place. In early OA, before cartilage degradation occurs, the thickness of the SB plate is decreased due to an elevated rate of bone remodelling. At this stage, a drastic loss of rod-like trabeculae and mild thickening

of plate-like trabeculae in subchondral trabecular bone can be detected. In late OA, when degenerative changes are evident in AC, the thickness of the SB plate is increased, probably due to the activated 'repairing' system, and the subchondral trabecular bone becomes sclerotic.[17] Cells involved in the remodelling of the SB are osteoclasts, osteoblasts (OBs), osteocytes as well as their corresponding progenitor cells: mononuclear cells and mesenchymal stem cells (MSCs). In addition, when vascularisation and innervation take place, endothelial cells (ECs) and nerve cells also interrupt the integrity of SB. Moreover, there is a tight connection between the maturation status of ECs and bone growth activity: when ECs of SB are in an activated, highly angiogenic state characterised by high expression of PECAM1 (CD31) and endomucin (EMCN), so-called type H vessels, they are surrounded by OBs and promote SB growth.[18] In turn, mechanical forces trigger the secretion of dentin matrix protein 1 from OBs, which inhibits vascular endothelial growth factor (VEGF) signalling and EMCN expression in ECs. This transforms type H vessels into quiescent type L endothelium and, thus, limits angiogenesis and bone growth activity.[19] Also osteoclasts participate in the development of arthritic bone diseases.[20] Peripheral blood mononuclear cells from patients with OA generated more osteoclasts and showed stronger bone resorption capacity.[21] Furthermore, the SB plate of patients with OA also harboured increased numbers of osteoclasts.[22] Increased osteoclast activity results in the loosening of bone extracellular matrix (ECM) and the release of embedded growth factors, such as TGFβ. TGFβ contributes to angiogenesis, nerve innervation and recruitment of MSCs.[23 24] These cellular and molecular activities together lead to the disruption of the SB architecture and impair its mechanical properties, such as load dissipation, and thus impairs AC homeostasis. A more detailed understanding of the cellular and molecular mechanism of SB remodelling in OA, especially in early OA, may provide valuable insights for the design of therapies to tackle OA even at an early stage.

In the last decade, efforts to identify mechanisms responsible for the development of OA are underway, using advanced technologies. Single-cell RNA sequencing (scRNA-seq) technology is an unbiased approach to explore the cellular heterogeneity in complex tissues, by revealing an individual transcriptome profile for each single cell, with high resolution and accuracy.[25 26] ScRNA-seq has been successfully applied to various types of joint tissues, such as AC,[27–29] meniscus,[30 31] synovial membrane[32 33] and even bone marrow microenvironment.[34] These studies have enhanced our knowledge on joint tissues in terms of cell-type heterogeneity, transcriptional diversity and OA-related pathogenesis. Particularly in AC, scRNA-seq analysis revealed substantial heterogeneity among chondrocytes, as reflected by seven different subpopulations. Furthermore, novel markers for cartilage progenitor cells were identified, which might prove helpful on the way towards cartilage regeneration.[27] Also in meniscus tissue, which comprised chondrocyte-like and fibroblast-like cells, seven clusters, including meniscus progenitor cells, were identified by scRNA-seq analysis.[30] With regards to SB, although two previous studies have, via scRNA-seq technology, looked at the cellular composition of total human hip femur heads,[35 36] specific investigations of the SB of knee joints in patients with OA are lacking.

In a current study published in *RMD Open*, Hu *et al*[37] analysed the SB of tibial plateaus that were removed from two patients with OA. Through unbiased clustering, 10 clusters were identified. These include immune cells: T cells, B cells, NK cells, NKT cells, dendritic cells and monocytes and macrophages as well as bone-related cells: (ECs; PECAM1$^+$), (OBs, RUNX2$^+$/CDH11$^+$), (MSCs, MCAM$^+$) and a mixture of osteoclasts, nerve cells and others. Following the detection of tight connections among ECs, OBs and MSCs, the authors further clustered the total bone-related cells and produced seven major cell clusters: precursor ECs (PreECs; C2CD4B$^+$/B3GNT5$^+$); ECs (VWF$^+$/KDR$^+$); endothelial OBs (EnOBs; ABCA10$^+$/MGST1$^+$); stromal OBs (StOBs; PTGS2$^+$/GFPT2$^+$); mineralised OBs (MinOBs; WIF1$^+$/NDNF$^+$) and two MSC subpopulations (figure 1). Further analysis revealed that Pre-ECs had a profile distinct from ECs, as they were enriched for genes involved in ribosome synthesis, exosome synthesis and inflammation signature, with high expression of C2CD4B and B3GNT5, whereas ECs were primarily enriched in genes related to angiogenesis, such as VWF and KDR. Similarly, OBs were further clustered into three differentially associated subpopulations: EnOBs, StOBs and MinOBs. GO and KEGG analyses point to a particularly high involvement of EnOBs in EC migration, VEGF binding, and the PDGFR-β signalling pathway, suggesting that this cluster may potentially affect angiogenesis. StOBs were enriched for collagen-related and fiber-related biological processes, such as collagen fibril organisation, fibronectin binding and ECM binding. MinOBs distinctively expressed an ossification and bone mineralisation gene signature. These analyses revealed both the compositional complexity and functional diversity of cells in SB.

By comparing healthy lateral side (Ctrl) and destructed medial side (OA), the authors have also observed certain OA-associated changes in the EC and OB populations. During OA progression, the total EC cluster substantially increased. subpopulation-wise, compared with Ctrl group, PreECs of the OA samples showed stronger protein synthesis and inflammation, whereas the EC subpopulation was enriched for angiogenesis-promoting functions, such as blood vessel development, general EC differentiation and platelet-derived growth factor binding. With regard to OBs, the number of total OBs also increased during OA progression, and even more so than the ECs. Regarding the OB subpopulations, EnOBs of OA samples displayed stronger activity in angiogenesis and wound healing processes than the Ctrl samples. As expected, OA-StOBs were enriched for genes involved

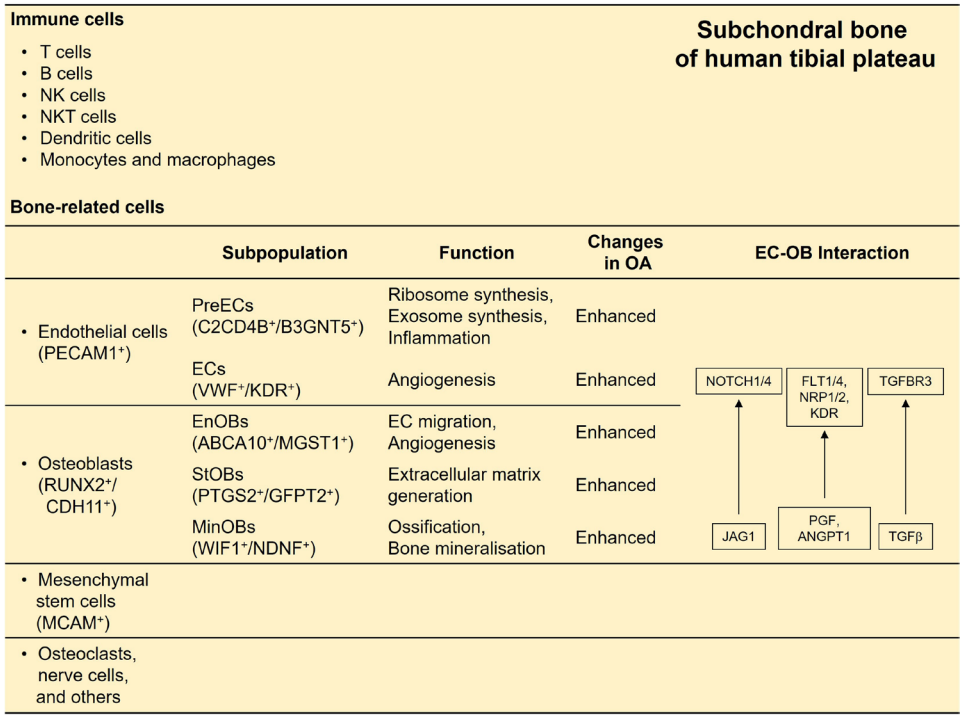

Figure 1 The composition, function and OA-associated changes of cells in human tibial subchondral bone. OA, osteoarthritis.

in ECM binding and collagen fibril organisation, and OA-MinOBs featured higher activity in the response to metal ions such as cadmium, copper and zinc. These results indicate that both EC-subpopulations and OB-subpopulations have intensified their designated functions during OA development.

The authors have also projected potential cell-cell interaction networks and indicated that EC subpopulation, but not PreECs, were the predominant cell population interacting with the OB subpopulations. ECs exhibited abundant expression of multiple membrane receptors for ligands important for vascular development, including NOTCH1, NOTCH4, VEGF receptors (KDR, FLT1, FLT4, NRP1, NRP2), TGFβ receptors (TGFBR3), which respectively bind to JAG1, the VEGF family, PGF, ANGPT1 and TGFβ ligand secreted by OB to promote angiogenesis. These results indicate that ECs are a mature EC subgroup with angiogenic function at the transcriptional level and are mainly coupled with OBs. While the analysis here is clear and convincing, the findings are somewhat at odds with a previous scRNA-seq study performed with human femoral head tissue, which revealed a close interaction between progenitor endothelial cells with osteoblastic lineage cells via the 'CLA2A1-ITGB1' coupling.[36] Thus, further analysis with proper control and cohort sample size is still required to clarify this discrepancy.

In summary, the current study has broken new ground, by introducing cellular constituents and crosstalk inside tibial SB of patients with OA using scRNA-seq technology, complementing the existing scRNA-seq–orientated studies that had focused on cartilage and synovial membrane of patients with OA. Of course, despite the novelty of this scRNA-seq study that has a specific focus on SB tissue, a critical limitation is that the control is the 'healthy' area of patients with OA but not material derived from really healthy, non-OA subjects. It is known that SB starts to undergo remodelling processes before evident cartilage degradation appears; therefore, the cells in the apparently 'healthy' tissue could have already started to go through pathologic changes at the molecular level when compared with non-OA individuals. Another limitation lies in the fact that there are only two samples in each group. In future studies, to achieve a more representative observation, more subjects should be included. Moreover, given the important contribution of osteoclasts to SB remodelling and OA development, it appears worthwhile to analyse the osteoclasts in detail, which has not been done in the current study. Finally, experimental evidence to support the predicted interactions, for example, in EC-OB cocultures, would be of high value and remains to be provided by future studies.

In general, given all the emerging evidence of the importance of SB in OA development, it is time to investigate the impact of the SB–AC interaction on OA development. AC and SB are integrated through the osteochondral junction and affect each other biologically and mechanically.[38 39] Small molecules such as sodium fluorescein were shown to directly diffuse from SB to the AC.[40 41] Moreover, in the case of severe OA, newly generated vasculature was found to penetrate from SB to AC and facilitate the biochemical interactions between these two tissues.[42] Even though the tidemark and mineralised cartilage may not be permeable to larger molecules such as TGFβ, the SB had been found to regulate TGFβ activity in AC through mechanical signals.[11] TGFβ is secreted by chondrocytes and sequestered in

AC matrix by the latency-associated peptide (LAP). A conformational change of LAP can result in the release of TGFβ and thereby allows the binding of TGFβ to its receptor on the chondrocyte membrane.[43] Mechanical stimuli coming from SB have been reported to trigger the conformational change of LAP.[11] The distribution of TGFβ activity in AC is well aligned with the structural configuration of SB, and SB structural alterations lead to redistribution of mechanical stress in AC, which in return determines the pattern of TGFβ activation in AC. To further understand the impact of the SB–AC interaction on OA development, we suggest more in-depth studies on SB itself, SB–AC interactions, and the cell–cell interactions among chondrocytes, endothelial cells, osteoblasts and osteoclasts.

Finally, since OA is 'a disease of the joint as an organ', it would be exciting to build a comprehensive silicon 3D structure of a diarthrodial joint. This model could be based on scRNA-seq data from joint tissues including AC,[27–29] SB[37], meniscus,[30 31] synovium membrane[32 33] and even bone marrow microenvironment.[34] It could delineate the alterations in cellular composition and function from the healthy state to various OA stages at the single-cell level. Such a 'digital joint' could function as an encyclopaedia for us to understand the pathogenesis of OA development and hence allow the targeted design of preventive and therapeutic approaches.

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