Generating 3D-cultured organoids for pre-clinical modeling and treatment of degenerative joint disease

Dear Editor,

Human cell-based and personalized in vitro cartilage models are urgently needed for osteoarthritis treatment in pre-clinical regenerative medicine development. Cellular self-assembly and condensations of the appropriate stem cells could initiate the formation of transient tissue structures programmed for specific organogenesis processes. This recapitulation of developmental events has previously been demonstrated for the formation of cardiac, epithelial and liver organoids. However, there has been very limited progress in the development of human cartilage organoids for osteoarthritis (OA). Here, we describe the fabrication of functional bioengineered cartilage organoid suitable for OA treatment.

Briefly, agarose microwell inserts for formation of a high number of synovial mesenchymal stromal cell (SMSC) organoids with homogeneous size distribution were created as previously described by Leijten et al. 3D-cultured SMSC organoids were generated and phenotypically analyzed for potential applications in OA modeling and treatment (Fig. 1a). SMSCs self-assembled to form a stack of cells to attain a spheroid shape in the 4-week cultivation (Fig. 1b; Supplementary Fig. S1a), demonstrating compaction of the organoids with a confined actin cytoskeleton network (Fig. 1b). Chondrogenesis was defined for the organoids (Supplementary Fig. S1, b–e), showing significantly greater expression of chondrogenic markers SRY-related high mobility group box gene 9 (SOX9) and aggrecan (ACAN) compared to the 2D control group (Supplementary Fig. S1, b–d). Meanwhile, the organoid lysates yielded a cartilaginous matrix that stained positively for toluidine blue, indicative of the formed proteoglycan-rich, cartilage-like extracellular matrix (ECM) (Supplementary Fig. S2b). Lacuna formation, a typical sign of cartilage formation, was observed for SMSC-organoids after transplantation subcutaneously in nude mice (Supplementary Fig. S1f), which generated substantial amounts of glycosaminoglycan (GAG) in cartilage tissues. Immunohistochemical analysis showed that the cartilage generated by SMSC-organoids deposited rich GAG (Supplementary Fig. S1, g–h) and expressed abundant ACAN and type II collagen (Fig. 1h), confirming better committed chondrogenic lineage (Supplementary Fig. S1, i–j). These results suggest that SMSC-organoids could generate ectopic cartilaginous tissue in vivo, indicating its potential in cartilage regeneration and OA therapy.

Main difference of the organoid we generated is its 3D shape and its firm stacking of cells. We presume that cellular communication would be altered in 3D organoid with modulated miRNA profiles in 3D organoid to mediate cellular communication. We sought to explore the miRNA expression profiles with miRNA microarray on three SMSC organoids vs. three 2D cultured SMSC samples. miR-138 expression was most significantly downregulated in SMSC organoids (Fig. 1c–e), which was further validated with qRT-PCR and fluorescence in situ hybridization (FISH) (Fig. 1e, f). To explore the role of miR-138 in OA development, miRNA microarray was performed on three OA cartilage samples from clinical OA patients vs. three control samples from patients with traumatic amputation (Supplementary Fig. S2, a–b). miR-138 expression was significantly elevated in OA samples and further validated in another independent 12 OA samples vs. 6 controls. (Supplementary Fig. S2, c–d) Moreover, the expression of miR-138 in cartilage tissues from OA patients was correlated with the joint degeneration grade (n = 21; r = 0.73, p < 0.001; Supplementary Fig. S2e). No significant difference was observed between OA and controls with respect to miR-138 level in the synovial tissues (Supplementary Fig. S2f). These findings suggest that miR-138 might mediate the better chondrogenic properties of SMSC organoids and have cartilage-specific effects in OA development. Dysregulated mRNAs were also identified in SMSC organoids (Supplementary Fig. S3, a–c). Forkhead box C1 FOXC1 was identified as the target of miR-138 (Fig. 1g, Supplementary Fig. S3, d–e). To further confirm the functional interaction between miR-138 and FOXC1, we performed luciferase reporter assay analysis. Co-transfected FOXC1 Wild Type (WT) with miR-138 mimics in cultured primary human SMSC cells was significantly lower than relative luciferase reporter activity of cells transfected FOXC1-mut (mutant) with miR-138 mimics (Fig. 1g, h). This effect was further supported by gene expression in cultured SMSC organoids and OA cartilage tissues (Fig. 1i, j; Supplementary Fig. S3, f–g). These results validated FOXC1 as a direct target of miR-138.

To identify the altered downstream pathways mediated by miR-138/FOXC1 signaling in SMSC organoids, enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in SMSC organoids were analyzed. Hypoxia induced factor (HIF) signaling pathway was significantly enriched for SMSC organoids in KEGG pathways (Supplementary Fig. S4a). Moreover, miR-138 inhibitor activated the expression of another subunit of hypoxia inducible factor-3 (HIF3a) which was reported to attenuate the transcription ability of HIF1α on its downstream target genes. Furthermore, FOXC1 small interfering RNA had effects on ACAN, SOX9, matrix metallopeptidase 13 (MMP13), vascular endothelial growth factor A, hypoxia inducible factor-1α (HIF1α) and hypoxia inducible factor-3αa (HIF3αa) genes similar to the effects induced by miR-138 (Supplementary Fig. S4b, d). miR-138 regulates chondrogenesis and OA progression by targeting the FOXC1/HIF pathway. These results indicate that miR-138-mediated chondrogenesis in OA is primarily through the FOXC1/HIF pathway. miR-138 inhibition upregulated FOXC1 expression, and FOXC1 further suppressed HIF1α transcription and upregulated HIF3α expression (Supplementary Fig. S4, b–d). Meanwhile, HIF3α could attenuate the binding of HIF1α to the hypoxia-responsive elements (HRE) located within the
enhancer/promoter of hypoxia-inducible target genes and hence inhibits HRE-driven transcriptional activation (Fig. 1k).

SMSC-organoids demonstrated chondrogenic properties and generated ectopic cartilaginous tissues in vivo. Meanwhile, downregulation of miR-138 was also previously reported as a protective factor in OA development. In this case, we hypothesize SMSC organoid might be a novel OA therapy with its modulation of the miR-138/FOXC1/HIF axis. To determine whether SMSC organoids transplantation would reduce or reverse the progression of OA, intra-articular injection of GFP-labeled SMSC-organoids was performed for rats with ACLT surgery (Supplementary Fig S5, a–b). Compared to control group with severe cartilage erosion, osteophyte formation and synovial inflammation, local delivery of SMSC or its organoids remarkably protected the structure of joint cartilage as determined by histological assessments and proteoglycan depositions (Fig. 1l; Supplementary Fig. S5c). No significant SBP difference was observed in subchondral bone (Supplementary Fig. S5c).
Moreover, expression of MMP13 was significantly decreased by SMSC organoids transplantation, whereas an increase in ACAN expression was noted (Fig. 1), indicating the restoration of balance in chondrocyte anabolism and catabolism by SMSC organoids. In evaluating the modulation of the miR-138/FOXC1/HIF signaling axis in OA and SMSC organoids transplantation, whereas an increase in ACAN expression and higher expression of its target gene FOXC1 further suppressed HIF1α transcription and upregulated HIF3α expression, which attenuates the binding of HIF1α to its target genes and hence inhibits HIF1α transcription. I Histological assessments of joint cartilage with HE (1st row), safranin-O staining O (2nd row) and immunostaining for ACAN, MMP13, miR-138, FOXC1, HIF1α and HIF3α in different groups. Sham: no surgery group; ACLT: OA model group with anterior cruciate ligament transection; ACLT + MSC: only SMSC was injected for OA treatment; ACLT + organoid: SMSC organoids were injected for OA treatment.

ACKNOWLEDGEMENTS
This work was funded by the National Key R&D Program of China (No. 2018YFB1105600, No. 2018YFA0703000), China National Natural Science Funds (No. 51631009, No. 81802122), Chinese post-doctoral funding (No. 2019M661559) and the Funds from Shanghai jiao tong university for the Clinical and Translational Research Center for 3D Printing Technology.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41392-021-00675-4.

Ethics approval and consent to participate: This study collected tissue samples from human subjects and the study was approved by the ethics committee of Shanghai ninth hospital affiliated to Shanghai jiao tong University. Informed consents were obtained from the participants before the study.

Competing interests: The authors declare no competing interests.

Ye Sun1,2, Qiang Wu3, Kerong Dai2, Yongqiang You2 and Wenbo Jiang4
1Department of Orthopaedics, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China; 2Clinical and Translational Research Center for 3D Printing Technology, Shanghai Key Laboratory of Orthopaedic Implants, Department of Orthopaedic Surgery, Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China and 3Department of Nephrology, Affiliated Hospital of Nanjing Medical University, North District of Suzhou Municipal Hospital, Suzhou, China Correspondence: Ye Sun (sunye881005@163.com)

REFERENCES
1. Giobbe, G. G. et al. Extracellular matrix hydrogel derived from decellularized tissue structures enables endodermal organoid culture. Nat. Commun. 10, 5658 (2019).
2. Irie, Y., Mizumoto, H., Fujino, S. & Kajiwara, T. Development of articular cartilage grafts using organoid formation techniques. Transpl. Proc. 40, 631–633 (2008).
3. Leijten, J. et al. Bioinspired seeding of biomaterials using three dimensional microtissues induces chondrogenic stem cell differentiation and cartilage formation under growth factor free conditions. Sci. Rep. 6, 36011 (2016).

DATA AVAILABILITY
All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.
4. Yoshida, M. et al. The transcription factor Foxc1 is necessary for Ihh-Gli2-regulated endochondral ossification. Nat. Commun. 6, 6653 (2015).
5. Biniecka, M. et al. Dysregulated bioenergetics: a key regulator of joint inflammation. Ann. Rheum. Dis. 75, 2192–2200 (2016).