

ORIGINAL RESEARCH ARTICLE

Self-organizing vascularized subchondral bone organoids from stromal vascular fraction enable functional osteochondral interface regeneration

Tao Qian^{1†}, Jiazhou Wu^{2†}, Zexian Liu^{2,3†}, Aiyuan Wang², Yanbin Wu², Junli Wang¹, Hongyu Jiang², Zhengrui Zhou², Cheng Huang², Yazhou Li², Junming Zhang¹, Biao Ma¹, Yun Bai¹, Jialiang You², Endong Luo², Dingkai Wang², Ying He^{2*}, and Jiang Peng^{1,2*}

¹Jinzhou Medical University, Linghe, Jinzhou, Liaoning, China

²Institute of Orthopedics, Beijing Key Laboratory of Regenerative Medicine in Orthopedics, Key Laboratory of Musculoskeletal Trauma and War Injuries, PLA, The Fourth Medical Center of Chinese PLA General Hospital, Beijing, China

³Institute of Orthopedics, Graduate School, Chinese People's Liberation Army General Hospital, Beijing, China

*Corresponding authors: Jiang Peng (pengjiang301@126.com); Ying He (251032611@qq.com)

†These authors contributed equally to this work.

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Abstract

Osteoarthritis is closely associated with subchondral bone (SCB) degeneration; however, current models fail to adequately mimic its complex microenvironment. Here, we developed a self-organizing SCB organoid (SSBO) by co-culturing stromal vascular fraction (SVF) cells with decellularized cartilage extracellular matrix (CECM). SVF provided cellular heterogeneity, including adipose-derived stem cells (ADSCs), endothelial cells, pericytes, and macrophages, while CECM served as a native scaffold with tissue-specific cues. SSBO exhibited spontaneous spheroid formation, active cellular infiltration, and dynamic matrix remodeling. Compared to ADSC-only controls, SSBO showed enhanced cell viability, vascularization, collagen remodeling, and spatial organization. Immunostaining and quantitative real-time polymerase chain reaction analyses confirmed an endochondral ossification-like process, characterized by the sequential expression of *SOX9*, *COL2A1*, *RUNX2*, *COL1A1*, and *OCN*. *In vivo* implantation into immunodeficient mice demonstrated robust angiogenesis, bone-like tissue formation, and integration with host vasculature. Furthermore, in a mouse osteochondral defect model, SSBO significantly promoted repair, as evidenced by increased bone volume, improved trabecular architecture, and enhanced cartilage regeneration. Collectively, this study presents a novel strategy for constructing vascularized, immunomodulatory, and osteogenic SCB organoids, offering a promising platform for regenerative medicine and bone–cartilage interface repair.

Keywords: Stromal vascular fraction; Organoids; Tissue engineering; Subchondral bone

1. Introduction

Osteoarthritis (OA) is one of the most common degenerative joint diseases worldwide. It severely affects patients' quality of life.^{1,2} With the aging of the population, the incidence of OA continues to rise. It has become a major public health concern and a socioeconomic burden.³ Typical pathological features of OA include cartilage degeneration, abnormal remodeling of the subchondral bone (SCB), and synovial

inflammation.^{4,5} Over the years, OA was thought to affect articular cartilage primarily. However, recent studies have shown that SCB also plays a key role in OA onset and progression.^{6,7} Clinical and basic research suggest that abnormal SCB remodeling often occurs before cartilage degeneration. Changes in SCB structure and function can impair nutrient supply and mechanical support to the overlying cartilage. In addition, SCB may contribute to OA progression through abnormal signaling pathways.^{8,9}

Therefore, restoring the structure and function of SCB is considered a potential target for OA treatment. It may offer a new strategy for early intervention and disease reversal.¹⁰

SCB lies beneath the articular cartilage. It consists of a dense bone plate and a network of trabecular bone. SCB plays key biological and mechanical roles.^{11,12} It supports the cartilage and provides it with nutrients. It also acts as a hub for signal transmission and regulation at the bone–cartilage interface.^{13,14} The structure of SCB is highly complex. It includes bone-related cells, such as osteoblasts, osteocytes, and osteoclasts. It also contains endothelial cells, immune cells, and stromal cells. Together, they form a dynamic “bone–vascular–immune” system.^{14–16} This system is essential for joint homeostasis, bone remodeling, blood flow regulation, and immune response. In diseases such as OA, an imbalance in the SCB microenvironment can lead to trabecular disruption, abnormal blood vessel growth, and increased local inflammation. These changes form a vicious cycle that worsens cartilage damage and speeds up disease progression.^{17–19} Although tissue engineering has made progress in bone regeneration, current strategies still cannot fully mimic the complex microenvironment of SCB.¹²

In recent years, organoid technology has opened new avenues for modeling and regenerating complex tissues *in vitro*.²⁰ Organoids are three-dimensional (3D) tissue structures derived from adult stem cells or pluripotent stem cells. They are cultured under specific conditions to mimic the structure and function of real organs.²¹ Since intestinal organoids were first established in 2009, this technology has been widely applied in disease modeling, drug screening, and regenerative medicine for various organs, including the liver,²² brain,²³ and kidney.²⁴ The main advantage of organoids is their ability to recreate complex cell types, spatial organization, and functional features *in vitro*. This greatly improves the physiological relevance and application potential of traditional tissue models. It also offers a new way to mimic SCB's complex structure.²⁵ However, no current studies have developed SCB organoids (SSBO) for regenerative repair. In particular, there is still no 3D model that combines key functions, such as vascularization, bone remodeling, and immune regulation.

Stromal vascular fraction (SVF) is a heterogeneous cell population rich in various cell types. It includes adipose-derived stem cells (ADSCs), endothelial progenitor cells (EPCs), smooth muscle cells, and macrophages.²⁶ Studies have shown that EPCs in SVF have strong angiogenic capacity. They can rapidly form vascular networks in ischemic or hypoxic environments.²⁶ ADSCs and macrophages also support angiogenesis by secreting pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), thereby improving local microcirculation.²⁷ More importantly, SVF has immunomodulatory properties. Its endogenous cells

can secrete anti-inflammatory factors, thereby reducing inflammation and creating a favorable microenvironment for tissue repair.²⁸ Therefore, SVF not only helps establish a vascularized microenvironment but also provides nutritional and signaling support for osteogenic cell growth and differentiation. It is a promising cell source for building organoids with both vascularization and immune-regulation functions.

To better mimic the complex SCB and support multicellular assembly, this study used cartilage extracellular matrix (CECM) as the scaffold material for organoid construction. CECM is derived from decellularized cartilage tissue. It retains key matrix components such as collagen, glycosaminoglycans, and various endogenous bioactive factors. It shows good biocompatibility and tissue-inductive properties.^{29,30} Previous studies have shown that CECM provides stable physical support for stem cell adhesion, growth, and self-organization. Its natural matrix structure and biochemical signals also promote spatial organization and functional integration among cells. This helps recreate a physiologically relevant 3D microenvironment.^{31–33} Therefore, CECM is a promising scaffold for reconstructing the structure and function of the SCB region. It is an ideal biomaterial choice for *in vitro* modeling of SSBO.³⁴

Based on this rationale, we developed a patient-derived SSBO model by co-culturing SVF cells with CECM. According to established definitions, organoids are 3D structures derived from stem or progenitor cells. They exhibit self-organization, include multiple cell types, and recapitulate key structural and functional features of native tissue. Unlike conventional 3D spheroids, which are typically homogeneous and lack spatial organization, or scaffold-based constructs that require external signals and pre-designed patterns, SSBO formation in our system depended on the intrinsic heterogeneity of SVF and the bioactive properties of CECM. This combination enabled spontaneous self-organization, self-differentiation, and the emergence of spatially patterned functional domains. SVF contained a mixture of ADSCs, endothelial cells, pericytes, and immune-regulatory cells, whereas CECM provided native cartilage-derived structural and biochemical cues that promote regeneration. Together, these components supported assembly of a complex 3D structure without the need for added growth factors or engineered signaling environments. As a result, SSBO exhibited essential organoid characteristics, including cellular heterogeneity, layered architecture, and functional biomimicry. *In vitro*, SSBO developed a trabecular-like structure with vascular channels and demonstrated enhanced osteogenesis, angiogenesis, and immunomodulation. To evaluate the role of SVF cellular diversity, we established a control group by co-culturing purified ADSCs with CECM (designated as the AM group). We compared the two groups with respect to structural organization, vascularization, bone

regeneration, and immune regulation. In a murine model of SCB defect, SSBO showed improved tissue repair, highlighting its translational potential for regenerating the bone–cartilage interface. In conclusion, we propose a self-organizing strategy for constructing SSBO using SVF and CECM. This approach provides a novel organoid-based platform for osteochondral tissue regeneration.

2. Materials and methods

2.1. Isolation and culture of SVF and human ADSCs

The procedures for isolating and culturing SVF and ADSCs received ethical clearance from the Ethics Committee of the Fourth Medical Center, PLA General Hospital (Approval No. 2023KY088-KS001). Subcutaneous adipose tissue was harvested via liposuction from patients undergoing hip replacement surgery and processed immediately for SVF isolation. To isolate the intermediate fat layer, the lipoaspirate was centrifuged at 900 rpm for 60 s. A 5 mL aliquot of the adipose tissue was transferred to Tube 1 for subsequent cell counting, while the remaining adipose tissue was retained in Tube 2. Both tubes were added with 0.1 mg/mL Liberase collagenase (Roche, Switzerland) and incubated in a 37°C water bath for 45 min. Enzymatic activity was inhibited by adding a culture medium containing a serum substitute. This was followed by centrifugation at 1,500 rpm for 10 min to separate the components. The resulting cell pellet was then collected and resuspended in a small volume of serum substitute-based MSC-T4 medium. Finally, the suspension was filtered through a 100 µm cell strainer to remove any remaining debris. Cells collected from Tube 1 were lysed with red blood cell lysis buffer and counted to determine the total cell number in Tube 2. The cell suspension from Tube 2 was adjusted to a final seeding density of 300,000 cells per well in a 96-well U-bottom plate. Each well received 200 µL of culture medium.

In addition, cells from Tube 1 were seeded into multiple 75 cm² culture flasks and cultured at 37°C in a 5% CO₂ atmosphere. Once they reached a confluence of 80–90%, primary human ADSCs were harvested using trypsin and reseeded at the same cell density in 96-well U-bottom plates. The SVF and ADSCs were extracted from the same donor and analyzed in parallel to ensure consistency.

2.2. Cell composition detection

The cellular composition of SVF and primary ADSCs was characterized through flow cytometry. Briefly, the cells were centrifuged, after which the SVF and primary ADSC single-cell suspensions were adjusted to 10⁶/mL and separated into seven 1.5 mL tubes labeled A–G, with 0.2 mL/tube. Tube A contained a cocktail of primary antibodies against cluster of differentiation (CD)31, CD34, CD45, CD73, CD90, and CD146. Tubes B–F served as single-color compensation controls, each containing an antibody against CD31, CD34,

CD45, CD73, CD90, or CD146, respectively, whereas tube G contained phosphate-buffered saline (PBS), serving as the blank control. Next, live/dead dye was added to the cells, followed by incubation in the dark at room temperature for 30 min. This was followed by washing and centrifugation twice; the samples were analyzed using flow cytometry, and the data were processed using FlowJo software (Version 10.8.1), FlowJo LLC., United States of America [USA]). Each experiment was repeated 3 times.

The gating strategy is illustrated in Figure S1. Singlets were first gated using forward scatter area versus forward scatter height parameters, followed by selection of viable cells based on live/dead staining. CD45 expression was used to separate CD45⁺ leukocytes from CD45[−] stromal cells. Within the CD45[−] population, ADSCs were defined as CD90⁺CD73⁺, EPCs as CD34⁺CD31⁺, and pericytes as CD146⁺CD90[−]CD73[−]. CD45⁺ cells were collectively defined as leukocytes. Subpopulation percentages were calculated based on the total number of live singlet events.

2.3. Preparation of CECM microparticles

Fresh porcine articular cartilage was harvested and mechanically fragmented. A section of the fragmented cartilage was reserved and assigned to the native cartilage group without further treatment. The remaining cartilage fragments underwent repeated freeze–thaw cycles, using trypsin and nuclease. The resulting suspension was subjected to differential centrifugation to extract cartilage particles. Residual chemical agents were thoroughly removed through repeated washing with PBS. The cartilage particles were filtered through 180 µm and 300 µm sieves to prepare microparticles with a size range of 180–300 µm. The final CECM microparticles were sterilized using the cobalt-60 gamma irradiation and stored at 4°C until further use.

2.4. Histological and 4',6-diamidino-2-phenylindole staining

Cartilage particles, both untreated and decellularized, were immersed in a tissue fixative solution (Solarbio, China) for 30 min. After fixation, the samples were embedded and sectioned at 5 µm using a cryostat (CM1850, Leica, Germany). Hematoxylin and eosin (H&E) staining (Solarbio, China) was utilized to evaluate the overall tissue architecture and morphological features. To highlight the distribution of proteoglycans within the extracellular matrix (ECM), Alcian blue staining (Solarbio, China) was performed. The nuclei of residual or infiltrated cells were identified via 4',6-diamidino-2-phenylindole (DAPI) staining (Solarbio, China).

2.5. DNA quantification

DNA was extracted from the samples using the QIAamp DNA Mini Kit (Product No. 56304, Qiagen, Germany)

and then quantified using the Quant-iT PicoGreen dsDNA Quantitation Kit (Product No. P7589, Invitrogen, USA) following the manufacturer's instructions. Next, fluorescence intensity was measured at 480 nm excitation and 520 nm emission using a microplate reader (Infinite 200 Pro system, TECAN Group Ltd., Switzerland). The concentration of the DNA was determined from a standard curve.

2.6. Scanning electron microscopy

To ensure structural preservation, the CECM microparticles were immersed in 2.5% glutaraldehyde (Solarbio, China) for 24 h and subsequently washed 3 times with PBS. A graded ethanol series was used to dehydrate the samples. The morphology and surface features of the microparticles were then observed and imaged using scanning electron microscopy (SEM) (APERO 2S, Thermo Fisher Scientific, USA), following the guidelines provided by the manufacturer.

2.7. Construction of SSBO

To prepare SSBO, CECM microparticles were added to low-adhesion 96-well U-bottom plates containing previously seeded cells at a ratio of 1×10^5 cells/mg of microparticles, with 3 mg of microparticles per well. The cell cultures were incubated at 37°C at 5% CO₂ in complete MSC-T4 medium supplemented with a serum substitute. Half of the medium was replaced every 2 days. The formation of spheroids was monitored over the first 4 days post-seeding at the following time points: 0, 24, 48, 72, and 96 h. Organoid development in the two groups was examined on days 0, 7, 14, and 21, with Day 0 defined as the time point when compact spheroids had formed—that is, 96 h after initial seeding.

Day 14 was selected as the primary observation time point based on preliminary assessments of cell viability, morphological organization, and early matrix deposition. This time point also corresponds to a biologically relevant phase in endochondral ossification, during which vascularization and mineralization are typically initiated. Therefore, it was used as a representative stage for in-depth characterization of SSBO development.

2.8. Cytoskeleton and nuclear visualization

Organoids from both experimental groups were fixed and cryosectioned into 5 µm-thick slices. They were then stained with antibodies against α-tubulin (1:200; ab7291, Abcam, United Kingdom) and counterstained with DAPI (Invitrogen, USA) to visualize the cytoskeletal organization in organoids through immunofluorescence. Fluorescent signals were detected using a multispectral imaging system (MSP-REM-P1E-Vectra Polaris, Akova Biosciences, USA)

to characterize the cytoskeletal distribution and nuclear morphology.

2.9. Cell viability and apoptosis assessment

Cell viability within the organoid spheroids was assessed using the PrestoBlue™ Cell Viability Reagent (Invitrogen, USA) according to the manufacturer's instructions at day −4 (initial seeding), day 0 (compact spheroid formation), and days 7, 14, and 21 after spheroid formation. A volume of PrestoBlue equivalent to 10% of the culture medium (20 µL/well) was added to the organoid-containing plates, and the plates were incubated at 37°C with 5% CO₂ for 1 h. The supernatant was then collected, and fluorescence intensity was measured. Quantitative analysis was performed according to the manufacturer's protocol, and cell viability was normalized to SSBO at day −4.

In addition, organoids from both groups at days −4, 0, 7, 14, and 21 were fixed, cryosectioned into 5 µm slices, and subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining using a commercial kit (Beyotime, China) to detect apoptotic cells, following the manufacturer's instructions. Quantification of the apoptotic signal was performed using ImageJ (Version XX, National Institutes of Health, USA).

2.10. Transmission electron microscopy

After 14 days of spheroid formation in both experimental groups, samples were collected for further processing. The samples were fixed in 2.5% glutaraldehyde for 24 h at 4°C. Subsequently, they were rinsed with PBS and treated with 1% osmium tetroxide for 1 h to enhance membrane contrast. Dehydration was carried out through a graded ethanol series and acetone. Finally, the samples were embedded, sectioned into ultrathin slices, stained with uranyl acetate and lead citrate, air-dried, and examined using a transmission electron microscope.

2.11. Quantitative real-time polymerase chain reaction (qRT-PCR)

To measure gene expression during differentiation of both organoid types, qRT-PCR was performed using samples collected on days 0, 7, and 14 after spheroid formation. Expression of chondrogenic markers (*SOX9*, *COL2A1*), osteogenic markers (*COL10A1*, *RUNX2*, *COL1A1*, *OCN*), inflammation-related markers (*IL1B*, *IL10*), and angiogenic markers (*VEGF*, *CD31*) was quantified. Primer sequences are listed in Table S1.

Total RNA was isolated from organoids at days 0, 7, and 14 using a total RNA isolation kit (RC112-01, Vazyme, China). The RNA was reverse-transcribed into cDNA using the HiScript® RT kit (R333-01, Vazyme, China). qRT-PCR amplification was performed with the SYBR®

qPCR Master Mix (Vazyme, China) on a real-time PCR system (QuantStudio 5, Thermo Fisher, USA). Finally, the expression of target mRNA was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to *GAPDH*, the internal control.

2.12. Osteogenic induction and alkaline phosphatase/alizarin red S staining

SSBO and AM groups were transferred to osteogenic induction medium on day 4 post-spheroid formation. Half of the medium was replaced daily. Samples were collected at days 7, 14, and 21. Following treatment with 4% paraformaldehyde and immersion in 30% sucrose for cryoprotection, the specimens were embedded in optimal cutting temperature compound for cryosectioning and cut at 5 μ m for subsequent staining. Each group is repeated 3 times at each time point. Quantitative analysis of staining intensity was normalized to the positive signal of the control AM group at day 7.

In alkaline phosphatase (ALP) staining, ALP activity was detected using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) staining kit (C3206, Beyotime, China). Samples were incubated with the working solution at room temperature in the dark for 5–30 min until color development. After staining, sections were rinsed with distilled water, mounted using neutral resin, and imaged using a panoramic confocal digital slide scanner.

The calcium deposition was assessed using Alizarin Red S staining. Samples were incubated with Alizarin Red S solution at room temperature for 10 min, followed by three washes with PBS. After dehydration and mounting with neutral resin, images were acquired using a panoramic confocal digital slide scanner.

2.13. Immunofluorescence staining

The organoids from both groups were fixed and cryosectioned into 5 μ m-thick slices. The overall structure and protein expression profiles during organoid differentiation at days 0, 7, and 14 were assessed using the Seven-Color Multiplex Fluorescence Staining Kit Plus (PTSA-67, Boster Biological Technology, China), following the manufacturer's instructions. The following primary antibodies were used: CD44 (1:200; ab254530, Abcam, UK), CD68 (1:200; ab955, Abcam, UK), CD45 (1:200; ab40763, Abcam, UK), platelet-derived growth factor receptor (PDGFR) (1:200; ab32570, Abcam, UK), CD31 (1:200; ab9498, Abcam, UK), sex-determining region Y-box 9 (SOX9) (1:200; ab185966, Abcam, UK), collagen type II alpha 1 chain (COL2A1) (1:200; NB600-844, Invitrogen, USA), collagen type X alpha 1 chain (COL10A1) (1:200; 14-9771-82, Invitrogen, USA), interleukin (IL)-1 β (1:200; ab2105, Invitrogen, USA), IL-10 (1:200; ab133575, Invitrogen, USA) and collagen type I alpha 1 chain

(COL1A1) (1:200; ab138492, Abcam, UK).

2.14. In vivo implantation and regenerative tissue evaluation

Male BALB/c nude mice with immunodeficiency were used in this study ($n = 8$). Six-week-old mice were randomly assigned to two experimental groups: SSBO and AM groups ($n = 4$ /group). Anesthesia was induced through intraperitoneal injection of 0.3% sodium pentobarbital. A dorsal midline incision approximately 1 cm in length was made, and two subcutaneous pockets were formed through blunt dissection techniques. The pre-fabricated organoid constructs were carefully inserted into these pockets, and the skin was closed using 6-0 surgical sutures (Ethicon, USA). After a 3-week implantation period, the animals were euthanized, the implanted materials were retrieved, fixed overnight in 4% paraformaldehyde, and subjected to subsequent histological and molecular analyses, including H&E staining, Masson's trichrome staining, immunofluorescence, and immunohistochemistry.

In a separate set of experiments, 8-week-old male BALB/c nude mice were randomly distributed into four groups ($n = 4$ each): Control (untreated), AM, SSBO, and Sham. Mice were anesthetized with 0.3% sodium pentobarbital before surgery. A medial skin incision was made to access the knee joint, exposing the patellar tendon and medial collateral ligament. This was followed by an incision in the joint capsule, and the knee was gently flexed to reveal the femoral trochlear groove. In the Sham group, the patella was temporarily dislocated and repositioned, followed by layered closure of the capsule, muscle, and skin. For the remaining groups, a standardized osteochondral defect was created by vertically inserting a 0.8 mm diameter round-tipped needle—with a positioning bead placed 1 mm from the tip—into the trochlear groove and rotating it gently. Organoid constructs were then implanted into the defect sites in the AM and SSBO groups, while the Control group received no intervention. All incisions were closed in layers. After 4 weeks, mice were euthanized, and their femurs were collected for gross morphological evaluation. The specimens were subsequently fixed overnight in 4% paraformaldehyde and analyzed using micro-computed tomography (Micro-CT), immunohistochemistry, and various histological stains, including H&E, Masson's trichrome, and Safranin O.

All procedures involving animals were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Yuma Bio Co., Ltd. (Beijing, China) under protocol number SYXK20250023.

2.15. Statistical analysis

All statistical analyses were performed using GraphPad Prism (v10, Dotmatics, UK). Quantitative data are presented as mean $\pm$ standard deviation from at least three independent biological replicates. Data normality was assessed using the Shapiro–Wilk test. For comparisons between two groups, the unpaired two-tailed Student's *t*-test was used for normally distributed data, while the Mann–Whitney *U* test was applied otherwise. For multiple group comparisons, one-way analysis of variance followed by Tukey's *post hoc* test was used for normally distributed data; otherwise, the Kruskal–Wallis test was applied. A $p < 0.05$ was considered statistically significant. Exact *p*-values are reported in the results section where applicable.

3. Results

3.1. Development of organoids

Freshly isolated human SVF cells and *in vitro*-expanded primary ADSCs were analyzed using flow cytometry to identify cellular subpopulations and measure their relative proportions (Figure S1A and S1B). Scatter diagrams of flow cytometric results (Figure 1A and B) and flow cytometric quantitative analysis (Figure S1C) demonstrated that the proportion of adipose-derived mesenchymal stem cells (CD45[−]CD73⁺/CD90⁺) was significantly enriched in the cultured ADSCs ($99.37 \pm 0.12\%$) compared to their proportion in SVF ($23.15 \pm 11.08\%$) ($p < 0.0001$). The proportions of EPCs (CD45[−]CD31[−]/CD34⁺) and pericytes (CD45[−]CD73[−]/CD90[−]/CD146⁺) in cultured ADSCs were $0.03 \pm 0.03\%$ and $0.00 \pm 0.00\%$, respectively, which were both lower than the corresponding values in the SVF ($1.36 \pm 1.15\%$ and $1.42 \pm 0.62\%$, respectively). Despite observable variation, the results did not demonstrate statistical significance. Notably, the leukocyte population (CD45⁺) was significantly reduced in cultured ADSCs ($0.27 \pm 0.14\%$) relative to SVF ($69.1 \pm 8.29\%$) ($p < 0.0001$).

The porcine cartilage was decellularized, ground, and sequentially filtered through 180 μm and 300 μm mesh filters to obtain CECM microparticles with particle sizes ranging from 180 to 300 μm . Results of the histological and DAPI staining indicated that the decellularization was successful, as no visible nuclei were seen within the lacunae of the CECM group relative to the native cartilage group, while the majority of the ECM was retained (Figure S2A). DNA quantification also indicated successful nuclear removal (Figure S2B) ($p < 0.0001$). Analysis of SEM images revealed that the surfaces of the CECM microparticles were porous and rich in collagen fibers (Figure S2C). Collectively, these results confirm that the CECM microparticles possess the potential to support cell adhesion and facilitate intercellular interactions. Next, the cells and CECM were seeded into low-adhesion 96-well plates for co-culture. The cells formed tight envelopes around the CECM and

self-assembled into compact, spheroidal aggregates within 4 days (Figure 1C).

To evaluate the morphological characteristics of the SSBO, microtubule staining was conducted (Figure 1D). The results indicated that the organoids exhibited a well-defined spherical morphology, with microtubules enriched along the luminal surface forming continuous ring-like structures. The contours of the organoids appeared smooth and intact, with no signs of collapse.

3.2. Analysis of organoid viability and structural features

To evaluate cell viability within the organoids, a PrestoBlue assay was performed for quantitative analysis (Figure 2A). The results showed that the AM group exhibited higher metabolic activity at the early stage of culture, likely due to their strong proliferative capacity during the prior two-dimensional culture. However, after transfer to the 3D environment, their viability declined, with a brief increase observed between days 7 and 14. In contrast, the SSBO group showed lower initial metabolic activity, possibly due to recent enzymatic digestion. However, viability recovered rapidly from day 0 after spheroid formation and remained consistently higher than that of the AM group. By day 21, both groups showed a decline in viability. Overall, SSBO maintained significantly higher cell viability throughout the culture period, suggesting more potent biological activity. TUNEL staining further confirmed these findings (Figure 2B and C). Although apoptosis increased over time in both groups, the SSBO group consistently showed a lower proportion of apoptotic cells, supporting its superior cell viability.

Hematoxylin and eosin staining was performed on day 14 organoids (Figure S3A). In the AM group, cells were mainly distributed on the outer surface of the CECM particles, with limited infiltration into the interior. The structure of the CECM remained largely intact. In contrast, the SSBO group showed tighter integration between cells and the CECM. A large number of cells migrated into the matrix, accompanied by noticeable degradation of the CECM. To further investigate the interaction between cells and the CECM, transmission electron microscopy was performed on day 14 organoids. In the AM group, most cells were attached to the CECM surface without deep infiltration (Figure 2D).

In contrast, cells in the SSBO group penetrated into the interior of the CECM particles. Some cells exhibited pseudopodia-like protrusions that extended into the matrix, indicating active participation in scaffold remodeling rather than passive embedding, suggesting strong matrix-penetrating ability (Figure 2E). Notably, the CECM in the SSBO group showed extensive degradation

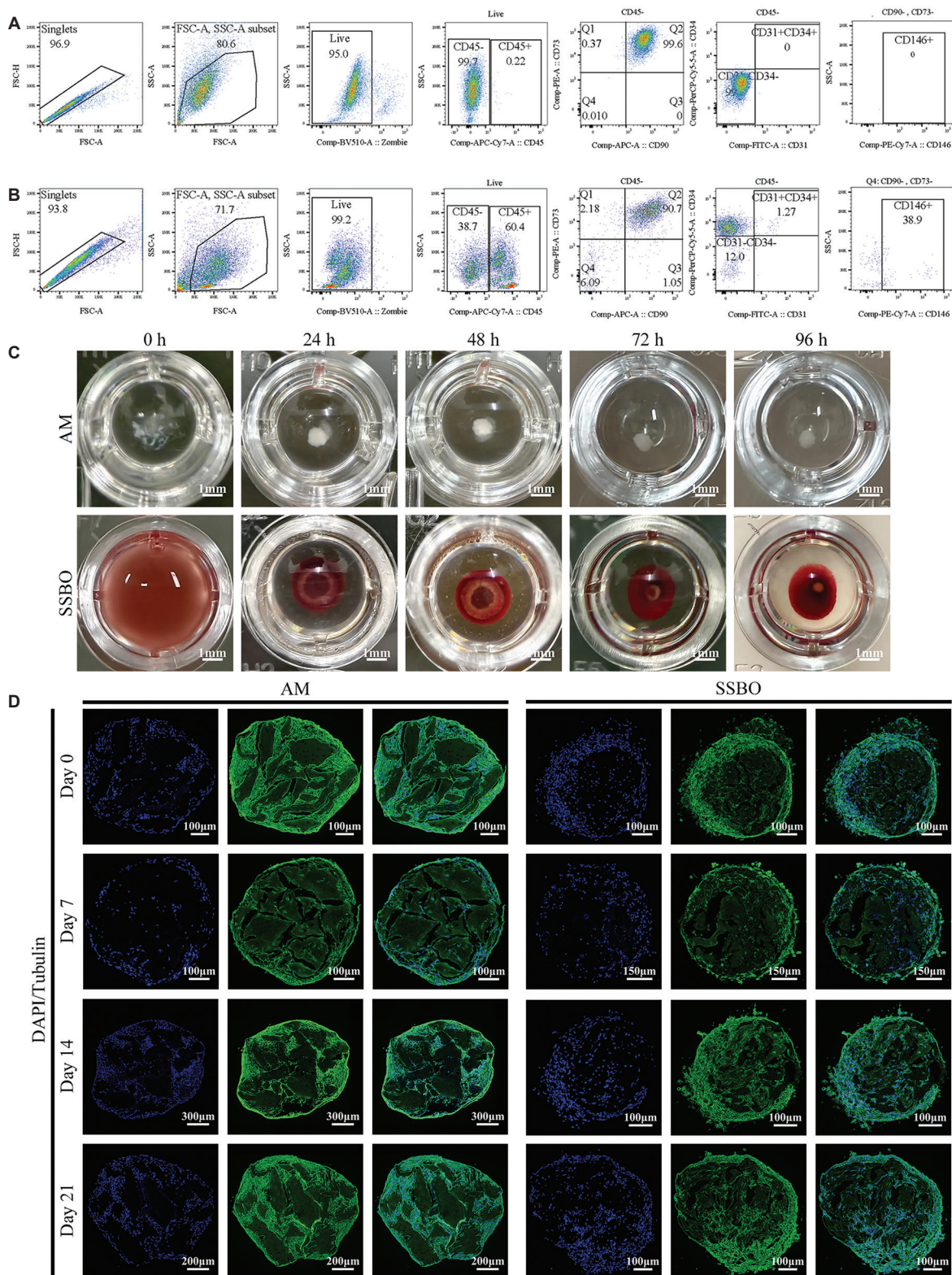


Figure 1. Development of organoids. (A) Flow cytometric analysis of primary human adipose-derived stem cells. (B) Flow cytometric analysis of stromal vascular fraction. (C) Gross morphology of organoids after co-culture. Scale bar: 1 mm. (D) Immunofluorescence staining of organoids showing structural organization. Scale bars: 100 μm, 150 μm, 200 μm, 300 μm; magnifications: 10×, 10×, 10×, 10×.

Abbreviations: AM: Control group (adipose-derived stem cells with cartilage extracellular matrix); DAPI: 4',6-diamidino-2-phenylindole; FSC-A: Forward scatter area; FSC-H: Forward scatter height; SSBO: Subchondral bone organoid; SSC-A: Side scatter area.

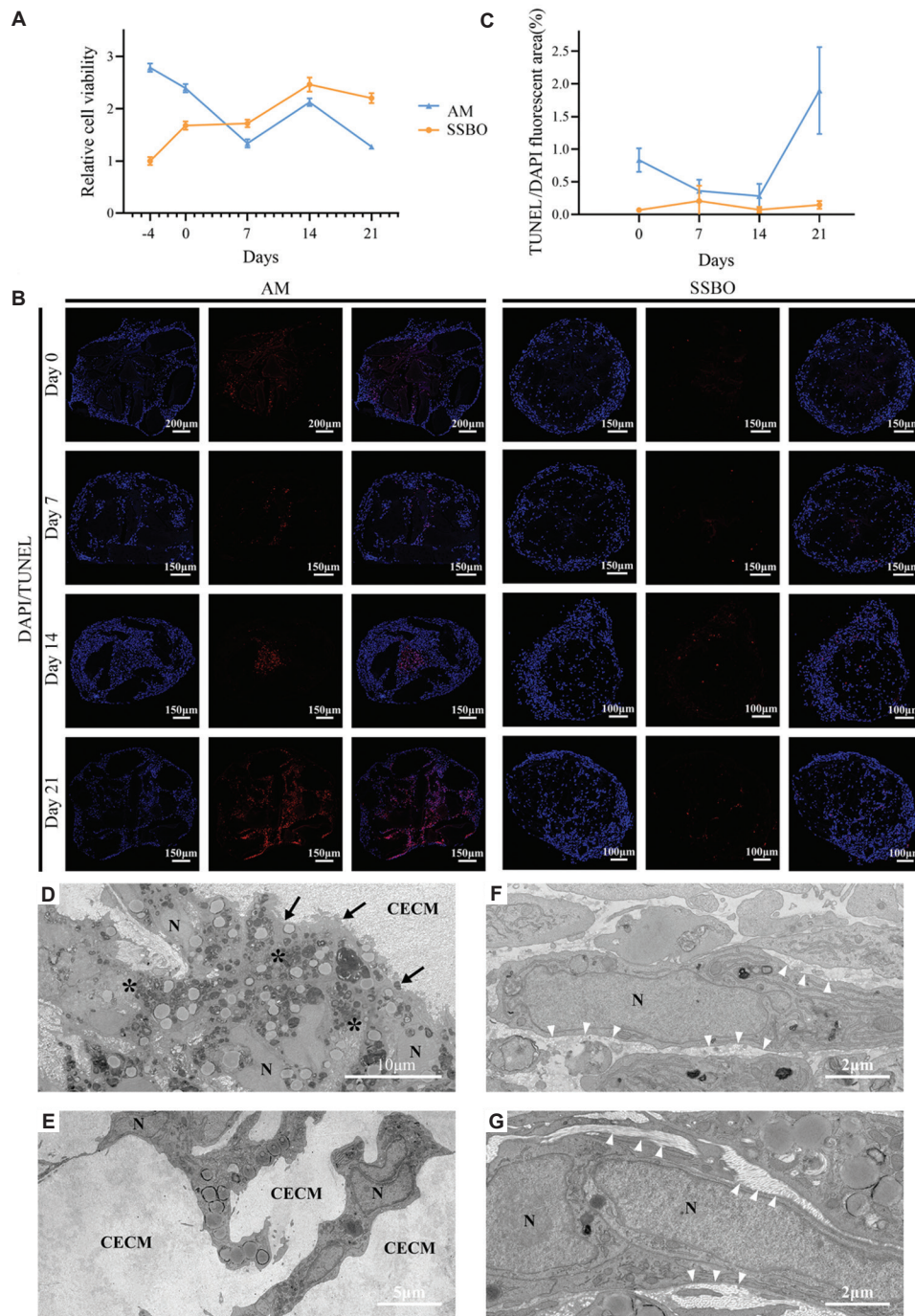


Figure 2. The vitality and structure of organoids. (A) Cell viability analysis using PrestoBlue assay. Values were normalized to the fluorescence signal of freshly seeded stromal vascular fraction cells, which served as the baseline reference due to equal initial cell seeding density across all groups. (B) TUNEL immunofluorescence staining to assess apoptosis. Scale bars: 100 μ m, 150 μ m, 200 μ m; magnifications: 10 $\times$, 10 $\times$, 10 $\times$. (C) Quantification of TUNEL⁺ signal normalized to DAPI fluorescence area. (D) and (F) Transmission electron microscopy images of the AM group after 14 days of culture. Scale bars: 2 μ m, 10 μ m; magnifications: 5 k $\times$, 1.5 k $\times$. (E) and (G) Transmission electron microscopy images of the SSBO group after 14 days of culture. Scale bars: 2 μ m, 5 μ m; magnifications: 5 k $\times$, 2 k $\times$.

Notes: *Nearby lysosomes; Black arrows indicate the boundary between the CECM and the cells; White arrowheads point to collagen fibers. "N" indicates the cell nucleus.

Abbreviations: AM: Control group (adipose-derived stem cells with cartilage extracellular matrix); CECM: Cartilage extracellular matrix; DAPI: 4',6-diamidino-2-phenylindole; SSBO: Subchondral bone organoid; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

and disorganized collagen fibers, with significantly lower collagen content compared to the AM group. This may result from the synergistic activity of multiple cell types in SSBO, which promotes the active degradation and utilization of the CECM. In addition, abundant collagen fibers were observed around the cells near the CECM in the SSBO group, markedly more than in the AM group (Figure 2F and G). Quantitative analysis showed that the SSBO group had a significantly higher collagen fiber density than the AM group ($p=0.0057$). The average fiber diameter was also larger in the SSBO group (Figure S3B). These observations suggest that SVF-derived cells may actively degrade and absorb key CECM components (such as collagen) and use them for ECM remodeling or biosynthesis. This mechanism may support cell differentiation, growth, and tissue repair—a hypothesis further verified in subsequent experiments. Moreover, cells in the AM group showed high lysosome content, suggesting a stressed or degenerative state. In contrast, SSBO cells contained abundant mitochondria and endoplasmic reticulum, with fewer lysosomes, indicating better cell function, higher metabolic activity, and overall improved biological performance (Figure S3C).

We performed quantitative analysis of organoid size on day 14 (Figure S3D). The results showed that the SSBO was smaller than the AM group. This may be due to two factors: (i) Higher initial cell viability in the AM group, leading to greater cell proliferation; and (ii) tighter integration between SVF cells and the CECM in the SSBO group. As a result, despite being seeded with the same number of cells, SSBO formed smaller organoids compared to AM.

3.3. Spatial organization and immunoregulatory characteristics of SSBO

To further characterize the cellular composition and spatial organization of SSBOs, we performed multiplex immunofluorescence co-staining at different time points (days 0, 7, and 14) (Figure 3A). At the early stage of spheroid formation (day 0), cells within SSBO were loosely arranged, lacking a clear tissue structure. As culture progressed, by day 14, the internal architecture became more organized. A dense, tubular network of EPCs was observed, primarily distributed around and along the surface of the CECM particles. The area of positive staining for these cells was significantly greater in the SSBO group than in the AM group, indicating stronger formation of a vascular-like structure in SSBO.

In contrast, AM organoids showed a simpler structure, mainly composed of scattered CD44⁺ ADSCs, with a larger positive staining area than in SSBO (Figure 3B and C), reflecting lower cellular heterogeneity. Although ADSCs remained the dominant cell type in SSBO, other non-ADSC cell populations were also present, indicating clear cellular heterogeneity. PDGFR⁺ pericytes were aligned in parallel

with EPCs but showed no co-expression. The number of pericytes was slightly higher than that of endothelial cells (Figure 3D). To further assess neovascularization within the organoids, we quantified CD31⁺ vascular structures from multiplex immunofluorescence images (Figure S4A). The SSBO group showed significantly greater total vessel length and the number of branch points than both the control and AM groups ($p<0.0001$). This indicates that the SSBO group developed a denser and more complex vascular network. Such vascularization is essential for scaffold integration and tissue repair.

Macrophages (CD68⁺ and CD45⁺) were primarily located at the periphery of the organoids, with a significantly higher abundance in the SSBO group compared to the AM group (Figure 3D). Notably, cells migrating into the CECM interior were mainly ADSCs and macrophages, suggesting their key roles in scaffold attachment, remodeling, and microenvironment regulation. In summary, SSBOs gradually developed a structured spatial distribution in 3D culture, exhibiting clear cellular heterogeneity and functional compartmentalization at the tissue level.

To delineate the immunomodulatory function of macrophages in SSBO assembly, expression of IL-1 β and IL-10 was evaluated through immunofluorescence analysis (Figure S4B). The results showed that macrophages in SSBO predominantly expressed IL-10, with the IL-10⁺ area significantly larger than that of IL-1 β (Figures S4C and S4D), suggesting a polarization toward an anti-inflammatory M2 phenotype. We also conducted qRT-PCR analysis of organoids at different time points (day 0, 7, and 14) (Figure S4E). In the SSBO group, *IL10* expression remained consistently high and was significantly elevated compared to the AM group throughout the culture period ($p<0.0001$). In contrast, *IL1B* expression peaked briefly at day 0 in the SSBO group, then rapidly declined to near-baseline levels. This indicates a transient pro-inflammatory response during early spheroid formation, followed by rapid suppression. Further analysis of the *IL10/IL1B* ratio revealed a significant increase in SSBO at day 7, while the AM group maintained a low ratio throughout. These findings suggest that macrophages in SSBO may help maintain immune homeostasis and a regenerative microenvironment by promoting anti-inflammatory cytokine expression and suppressing proinflammatory signals. In summary, macrophages in SSBO exhibit an IL-10-dominant immunoregulatory profile, which may play a key role in tissue repair, matrix remodeling, and microenvironmental stability.

3.4. In vitro endochondral ossification-like differentiation of SSBO

To dynamically track SSBO differentiation, we analyzed the expression of key lineage-related genes at days 0, 7, and 14

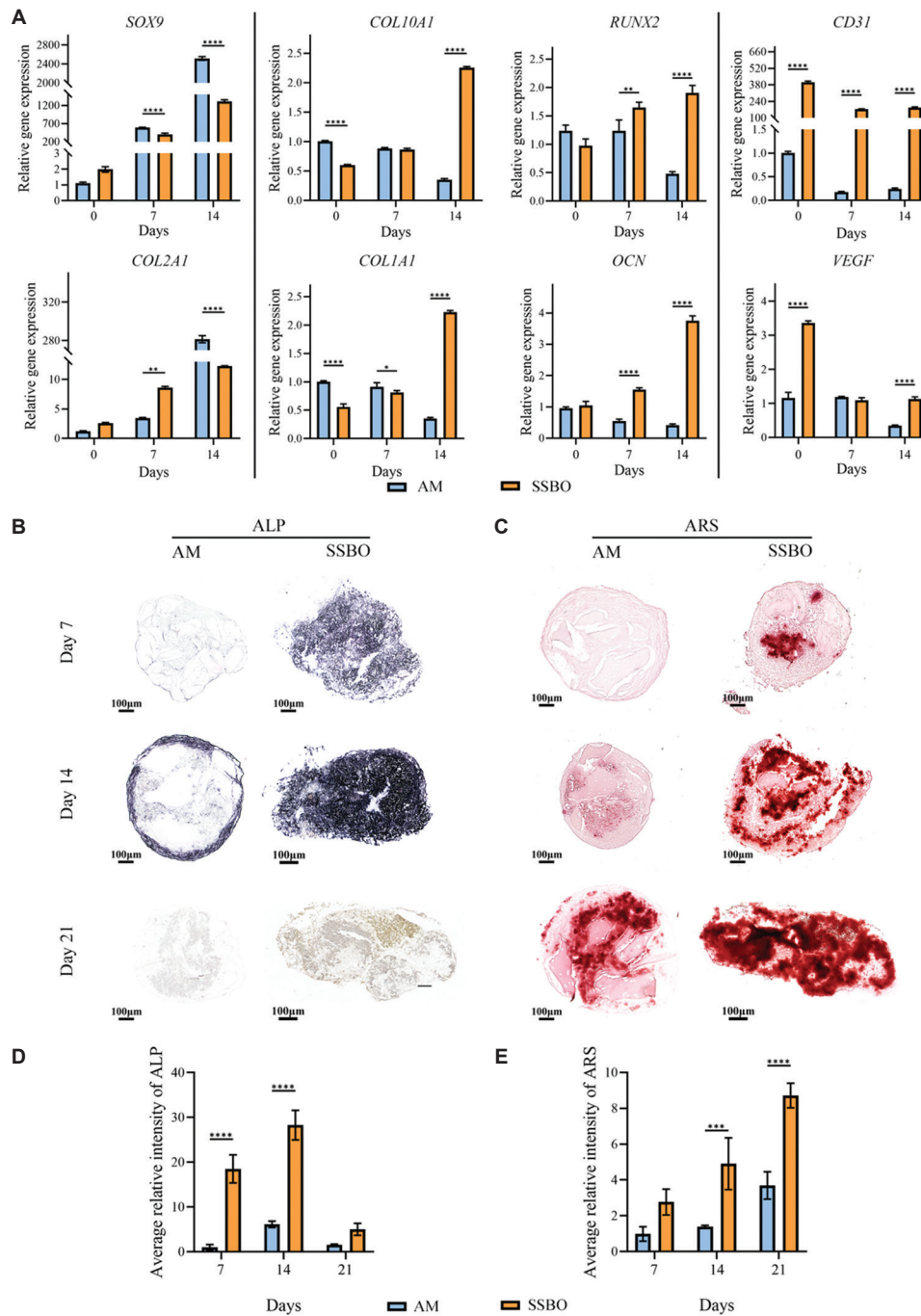


Figure 3. Spatial distribution of cells within the organoid. (A) Representative multicolor immunofluorescence images of SSBO and AM on days 0, 7, and 14 of culture. From left to right: nuclear staining (DAPI), adipose-derived mesenchymal stem cell marker (CD44), M0 macrophage marker (CD68), leukocyte marker (CD45), pericyte marker (PDGFR), endothelial cell marker (CD31), and merged images (Merge). Scale bars: 150 µm, 200 µm, 400 µm; magnifications: 10×, 10×, 10×. (B) High-magnification view of AM on day 14 of spheroid formation. Scale bar: 200 µm; magnification: 10×. (C) High-magnification view of SSBO on day 14 of spheroid formation. Scale bar: 200 µm; magnification: 10×. (D) Quantification of ALP-positive staining area (%). (E) Quantification of ARS-positive staining area (%).

Abbreviations: AM: Control group (adipose-derived stem cells with cartilage extracellular matrix); DAPI: 4',6-diamidino-2-phenylindole; PDGFR: Platelet-derived growth factor receptor; SSBO: Subchondral bone organoid.

(Figure 4A). Both SSBO and the control group AM showed a trend toward chondrogenic differentiation, as indicated by the time-dependent upregulation of significant cartilage

markers *SOX9* and *COL2A1*. Notably, *COL2A1* expression in the SSBO group was significantly higher than in the AM group at day 7, while *SOX9* expression decreased after

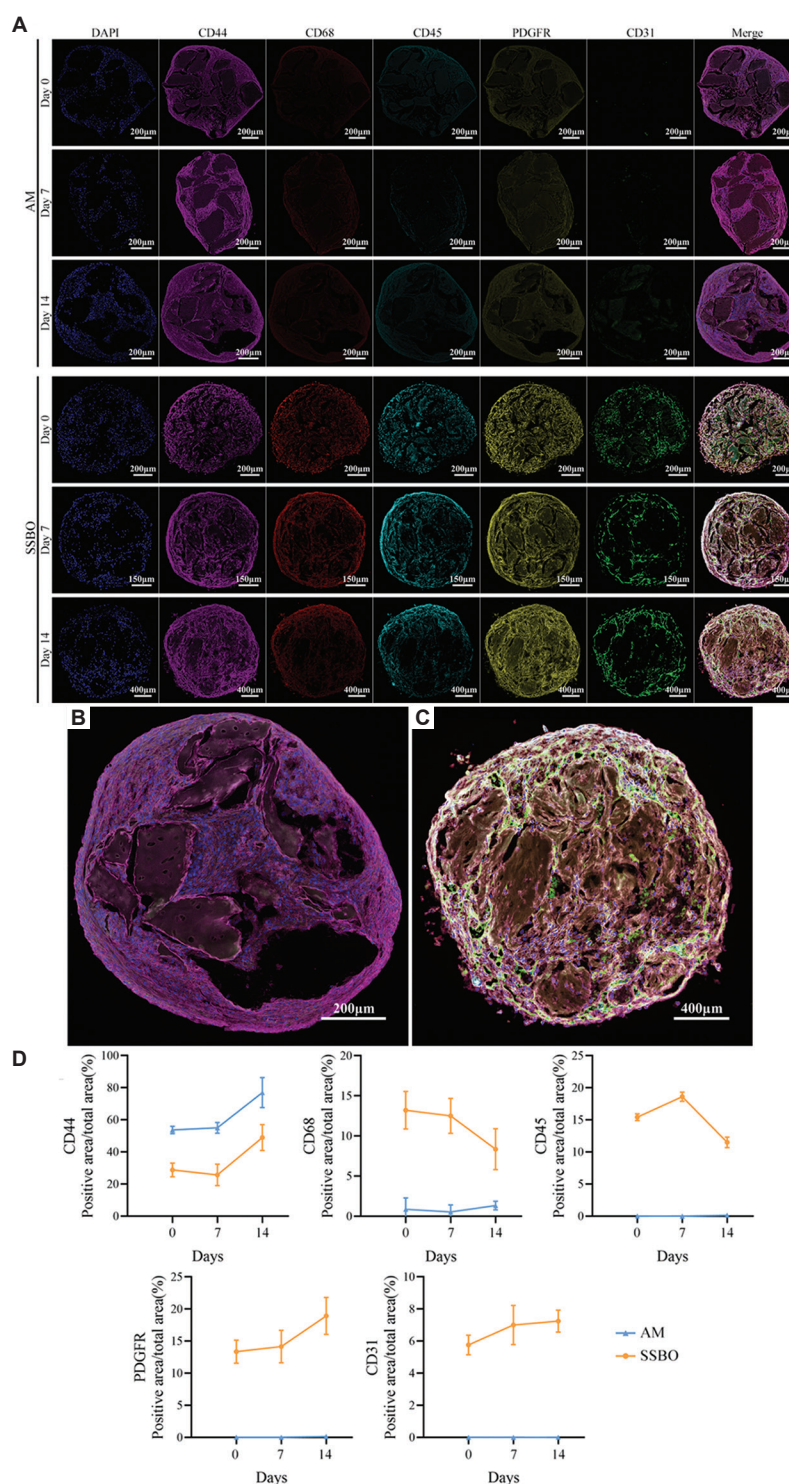


Figure 4. Differentiation of organoids. (A) Quantitative real-time polymerase chain reaction analysis of chondrogenic gene markers (*SOX9*, *COL2A1*), hypertrophic chondrocyte, and osteogenic markers (*COL10A1*, *RUNX2*, *COL1A1*, *OCN*), and angiogenic markers (*CD31*, *VEGF*) in both groups. Gene expression levels were normalized to the AM group at Day 0. (B) ALP staining of AM and SSBO at different time points during osteogenic induction. Scale bar: 100 μ m; magnification: 9 $\times$. (C) Alizarin Red S staining of AM and SSBO at different time points during osteogenic induction. Scale bar: 100 μ m; magnification: 9 $\times$. (D) Quantitative analysis of ALP staining normalized to AM on day 7 of osteogenic induction. (E) Quantitative analysis of ARS staining normalized to AM on day 7 of osteogenic induction.

Abbreviations: ALP: Alkaline phosphatase; AM: Control group (adipose-derived stem cells with cartilage extracellular matrix); ARS: Alizarin Red S; SSBO: Subchondral bone organoid.

an initial peak, falling below that of the AM group. This suggests that SSBO may have transitioned from early-stage chondrogenesis to a more mature cartilage state. Interestingly, by day 14, *SOX9* expression continued to rise in both groups, but remained significantly lower in SSBO compared to AM. Although this pattern does not fully align with the classical hypertrophic-phase dynamics of *SOX9* in endochondral ossification, the deviation may be attributed to sustained signaling from the CECM microenvironment that promotes maintenance of the chondrogenic phenotype. Meanwhile, expression levels of *COL10A1* (a hypertrophic cartilage marker), *RUNX2* (an osteogenic transcription factor), *COL1A1*, and *OCN* (markers of mature osteogenesis) increased markedly over time in SSBO, especially at day 14. In contrast, these genes showed a declining trend in the AM group, suggesting that AM differentiation may have stalled at a stable chondrogenic phase. Together, these results indicate that SSBO undergoes a coordinated transition from cartilage to hypertrophic cartilage and then to bone, consistent with an endochondral ossification-like pathway, which closely resembles the physiological process of SCB formation.

To further evaluate the angiogenic potential of SSBO, we analyzed the expression of vascular-related markers *VEGF* and *CD31* (Figure 4A). During the early stage (days 0, 7), both markers were downregulated in the SSBO group, possibly due to temporary suppression of vascular signaling by the CECM microenvironment. However, their expression began to increase from day 7, suggesting progressive activation of angiogenic signaling during tissue maturation and the cartilage-to-bone transition. This pattern reflects a process of vascular reconstruction or re-initiation. In contrast, throughout the entire induction period, the expression levels of *VEGF* and *CD31* in SSBO remained consistently higher than those in the AM group. The AM group, lacking vascular-related cell components, showed a continuous decline in both markers. These results indicate that the EPCs and pro-angiogenic factors enriched within SVF play a critical role in organoid assembly. They provide both structural and signaling support for the establishment of a vascularized microenvironment.

To further verify the osteogenic potential of the SSBO, osteogenic induction was performed for 0, 7, and 14 days, followed by ALP and Alizarin Red S staining (Figure 4B-D). The results showed that the SSBO group exhibited markedly higher ALP activity and more abundant calcium deposition, indicating significantly enhanced osteogenic capacity.

To further validate the differentiation process at the spatial level, multicolor immunofluorescence co-localization was performed to assess protein expression patterns in SSBO and AM organoids (Figure 5A). In the SSBO group, *SOX9* expression gradually decreased from

day 7, while *COL2A1*, *COL10A1*, and *COL1A1* expressions progressively increased. These findings confirm that SSBO undergoes a sequential differentiation process *in vitro*, progressing from chondrogenesis to hypertrophy and, finally, to osteogenesis. In contrast, the AM group showed only a slight upregulation of *COL2A1*, with no significant changes in *COL10A1* or *COL1A1*, suggesting that differentiation remained at the chondrogenic stage and lacked the capacity to progress toward osteogenesis. Furthermore, *CD31* immunostaining revealed the progressive formation of abundant and well-organized vascular-like networks in the SSBO group, with signal intensity increasing over time (Figure 5B-D). In the AM group, *CD31* expression remained minimal, and no similar vascular structures were observed. Interestingly, although *CD31* mRNA expression showed only a modest increase in qRT-PCR analysis, the protein exhibited pronounced spatial clustering and morphological organization within the tissue. This suggests that the formation of vascular-like structures in SSBO may depend more on cellular self-assembly and tissue remodeling rather than transcriptional upregulation alone. These observations further support the crucial role of EPCs within SVF in driving organoid self-organization and vascularization.

3.5. *In vivo* bone-like tissue formation and vascular integration of SSBO in immunodeficient mice

To evaluate the *in vivo* osteogenic potential of SSBOs and their ability to integrate with the host vasculature, four SSBOs, pre-induced *in vitro* for 14 days, were co-cultured for 24 h to promote fusion and form a stable, spheroid-like structure. These were then subcutaneously implanted into immunodeficient mice and harvested for analysis after 3 weeks (Figure 6A). Macroscopic observation revealed extensive vascular network formation around the SSBO implantation site, whereas AM organoids showed no visible vascular structures. This suggests that SSBOs possess superior angiogenic potential and a greater capacity to integrate with host vasculature. Histological analysis further supported these findings. H&E staining (Figure 6B) showed that SSBO implants maintained well-organized tissue architecture and uniform cellular distribution *in vivo*, with multiple vascular-like structures observed (Figure S5A). In contrast, the AM group showed a loose tissue structure with minimal vascularization. Masson's trichrome staining (Figure 6C) revealed abundant blue-stained collagen fibers surrounding the SSBOs, arranged in a regular pattern indicative of mature bone-like tissue formation. In contrast, the AM group showed minimal collagen deposition, suggesting a limited capacity for *in vivo* bone-like tissue reconstruction.

To further validate the osteogenic differentiation potential of SSBO, we examined the expression of the late-stage osteogenic marker osteocalcin (*OCN*).

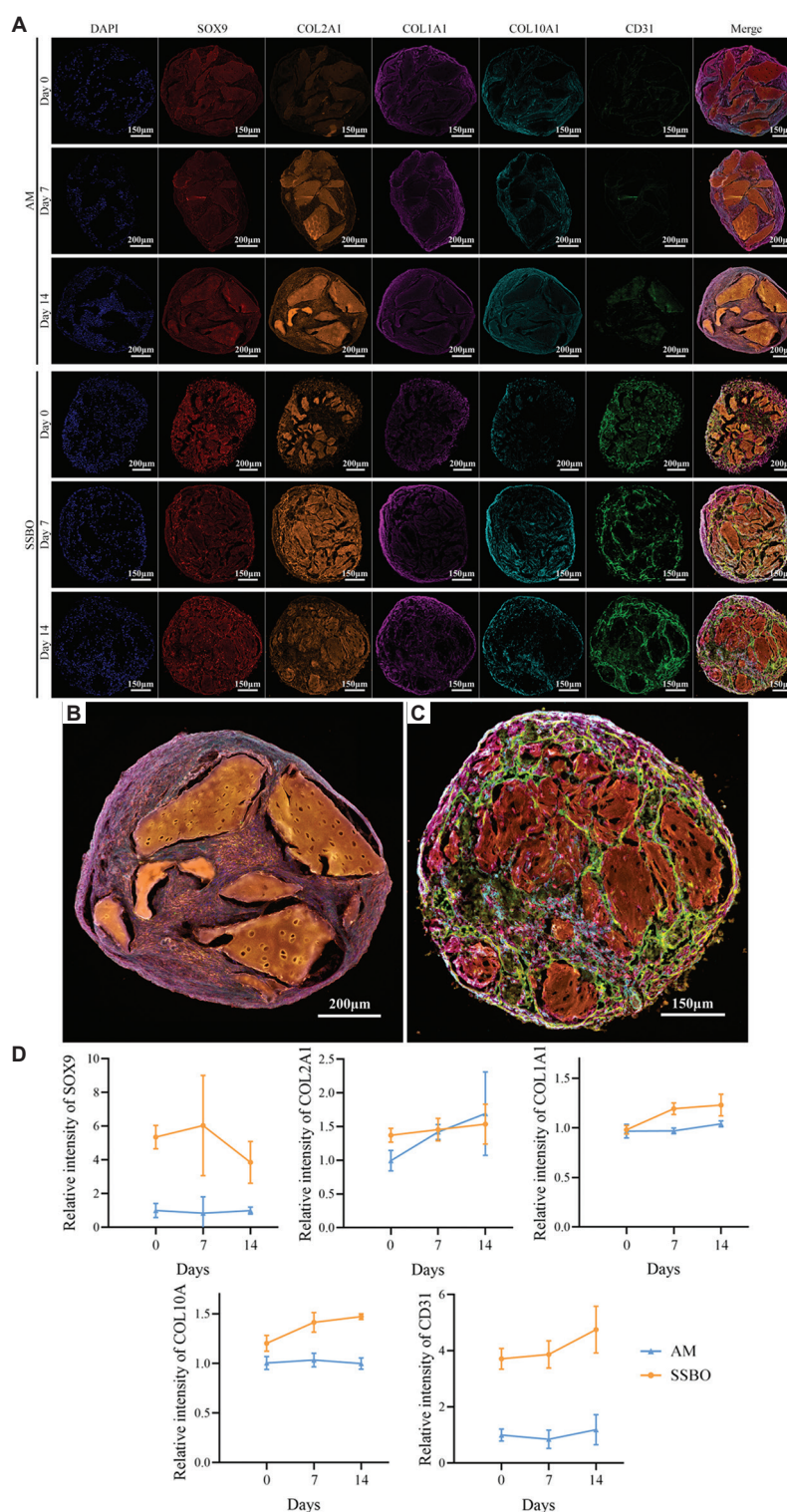


Figure 5. Differentiation of organoids. (A) Representative multicolor immunofluorescence images of SSBO and AM on days 0, 7, and 14 of culture. From left to right: Nuclear staining (DAPI), chondrogenic markers (SOX9, COL2A1), osteogenic markers (COL1A1), hypertrophic chondrocyte marker (COL10A1), endothelial cell marker (CD31), and merged images (Merge). Scale bars: 150 μ m, 200 μ m; magnifications: 10 $\times$, 10 $\times$. (B) High-magnification view of AM on day 14 of spheroid formation. Scale bar: 200 μ m; magnification: 10 $\times$. (C) High-magnification view of SSBO on day 14 of spheroid formation. Scale bar: 150 μ m; magnification: 10 $\times$. (D) Quantification of the relative intensity for each marker shown in (A). Abbreviations: AM: Control group (adipose-derived stem cells with cartilage extracellular matrix); CD: Cluster of differentiation; COL: Collagen; DAPI: 4',6-diamidino-2-phenylindole; SOX9: Sex-determining region Y-box 9; SSBO: Subchondral bone organoid.

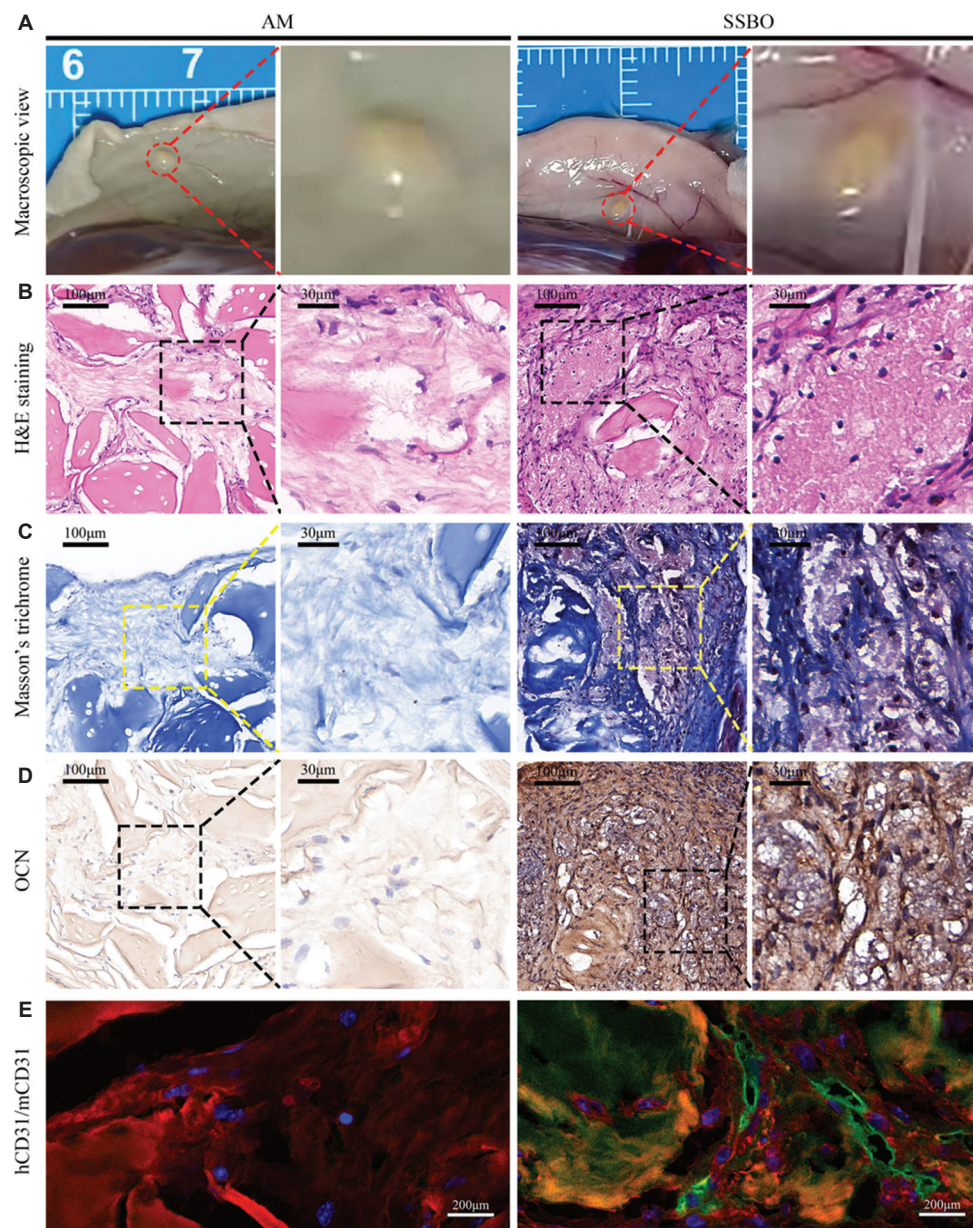


Figure 6. Evaluation of ectopic bone formation after subcutaneous implantation for 3 weeks. (A) Representative macroscopic views of tissue regeneration in each group following 3 weeks of subcutaneous implantation in bagg albino laboratory-bred/C (BALB/c) nude mice. (B) H&E staining of explanted tissues. Scale bars: 30 μm, 100 μm; magnifications: 20×, 60×. (C) Masson's trichrome staining. Scale bars: 30 μm, 100 μm; magnifications: 20×, 60×. (D) Immunohistochemical staining for OCN. Scale bars: 30 μm, 100 μm; magnifications: 20×, 60×. (E) Immunofluorescence staining for human and murine CD31 to assess vascularization. Scale bar: 200 μm; magnification: 40×.

Abbreviations: AM: Control group (adipose-derived stem cells with cartilage extracellular matrix); H&E: Hematoxylin and eosin; OCN: Osteocalcin; SSBO: Subchondral bone organoid.

Immunohistochemical staining (Figure 6D) revealed widespread and intense OCN⁺ signals in the SSBO implantation sites, whereas the AM group exhibited significantly lower expression levels (Figure S5B) ($p=0.001$). These findings collectively confirm that SSBOs possess robust osteoinductive capacity *in vivo*. They can effectively integrate with the host vascular system and initiate the

formation of bone-like tissue.

In addition, CD31 immunofluorescence staining (Figure 6E) confirmed *in vivo* vascularization within the organoid implants. In the SSBO group, a large number of human CD31⁺ cells were observed, along with mouse CD31⁺ cells, indicating the presence of chimeric vascular structures. These findings suggest that SSBO can recruit

and integrate into the host vascular system. In contrast, the AM group showed minimal murine CD31+ ingrowth and no observable vascular integration.

3.6. Osteochondral defect repair and SCB structure reconstruction in mice

To further evaluate the regenerative potential of SSBOs in bone tissue repair and osteochondral interface reconstruction, a mouse distal femoral osteochondral defect model was established. The defect measured approximately 0.8 mm in diameter and 1.0 mm in depth. Experimental animals were randomly assigned to four groups: Blank control, AM group, SSBO group, and sham-operated group. Samples were harvested from euthanized animals at 4 weeks post-surgery for evaluation.

Macroscopic observations showed that the blank control group exhibited a pronounced depression at the defect site, with a rough surface and slow repair progression. In the AM group, the defect was partially filled, but the surface remained irregular. In contrast, the SSBO group demonstrated complete coverage of the defect with regenerated tissue, a smooth surface, and a color closely matching the surrounding cartilage, indicating a favorable reconstruction outcome. Micro-CT analysis further confirmed these findings (Figure 7A). The SSBO group displayed substantial new bone formation, with the defect area nearly fully filled. The AM group showed partial filling of the defect, while the blank group exhibited minimal signs of healing. Quantitative analysis (Figure 7B) revealed that the bone volume to tissue volume ratio in the SSBO group was significantly higher than in the blank group. Although slightly lower than that of the sham-operated group, the difference was not statistically significant. Furthermore, the SSBO group demonstrated superior structural outcomes. Quantitative micro-CT analysis showed that bone mineral density, trabecular thickness, and trabecular number were higher than those in the AM and blank groups and approached the levels observed in the sham group.

Immunohistochemical staining (Figure 7C) revealed widespread and intense osterix (OSX)⁺ signals in the implantation region of the SSBO group, with expression levels significantly higher than those observed in the AM ($p=0.0028$) and blank control ($p=0.001$) groups, and comparable to the sham-operated group. Quantitative analysis further confirmed these findings: Although a difference remained between the SSBO and sham groups, the OSX expression in the SSBO group was notably higher than in the other groups (Figure S5C). These results suggest that SSBO possesses a significant advantage in promoting the maturation of SCB tissue.

Histological analysis further confirmed the experimental findings. H&E staining showed that the SSBO group

developed a large number of well-aligned newly formed bone trabeculae, and the regenerated osteochondral tissue closely resembled native architecture (Figure 8A). Masson's trichrome staining revealed abundant collagen fiber deposition in the SSBO group, indicating robust matrix reconstruction (Figure 8B). Safranin O staining demonstrated that the regenerated cartilage layer was uniform, continuous, and of appropriate thickness, comparable to the sham group (Figure 8C). Conversely, the Blank and AM groups were mainly filled with fibrous tissue, with discontinuous bone structures and incomplete or collapsed cartilage layers. To quantitatively evaluate the quality of osteochondral defect repair, histological sections were assessed using the O'Driscoll scoring system. The SSBO group exhibited significantly higher scores than both the control group ($p<0.0001$) and the AM group ($p=0.0025$), indicating a superior tissue regeneration outcome (Figure S5D). Taken together, the results demonstrate that SSBO facilitates SCB regeneration and supports the formation of structurally intact and morphologically appropriate articular cartilage. Notably, the findings further indicate that effective cartilage regeneration relies heavily on the functional restoration of the SCB.

4. Discussion

The SCB is a structurally complex and functionally active component of the joint microenvironment. It has been increasingly recognized for its critical role in the initiation and progression of OA.¹⁸ Beyond providing mechanical support and load buffering, SCB contributes to joint homeostasis by regulating osteochondral interface signaling, microvascular stability, and immune responses.^{17,35} However, most current organoid models focus on single tissue types and fail to recapitulate the multicellular, multi-functional microenvironment of SCB. The development of a 3D *in vitro* model that integrates angiogenic, immunomodulatory, and osteogenic functions remains a significant challenge in tissue engineering and disease modeling.³⁶⁻³⁸

In the current study, we successfully constructed an SSBO by leveraging the intrinsic cellular heterogeneity of SVF and a CECM scaffold. Unlike conventional approaches that rely on single-cell populations, such as ADSCs or bone marrow-derived mesenchymal stem cells, SVF comprises a diverse set of functional subtypes—including EPCs, mesenchymal stem cells, macrophages, and pericytes—endowing it with pro-vascular, mesenchymal, and immunoregulatory potential.³⁹⁻⁴¹ Flow cytometry confirmed the presence of these subpopulations, while histological and ultrastructural analyses demonstrated their active participation in scaffold colonization and tissue remodeling.

Beyond self-assembly, SSBO exhibited dynamic matrix remodeling driven by coordinated interactions between

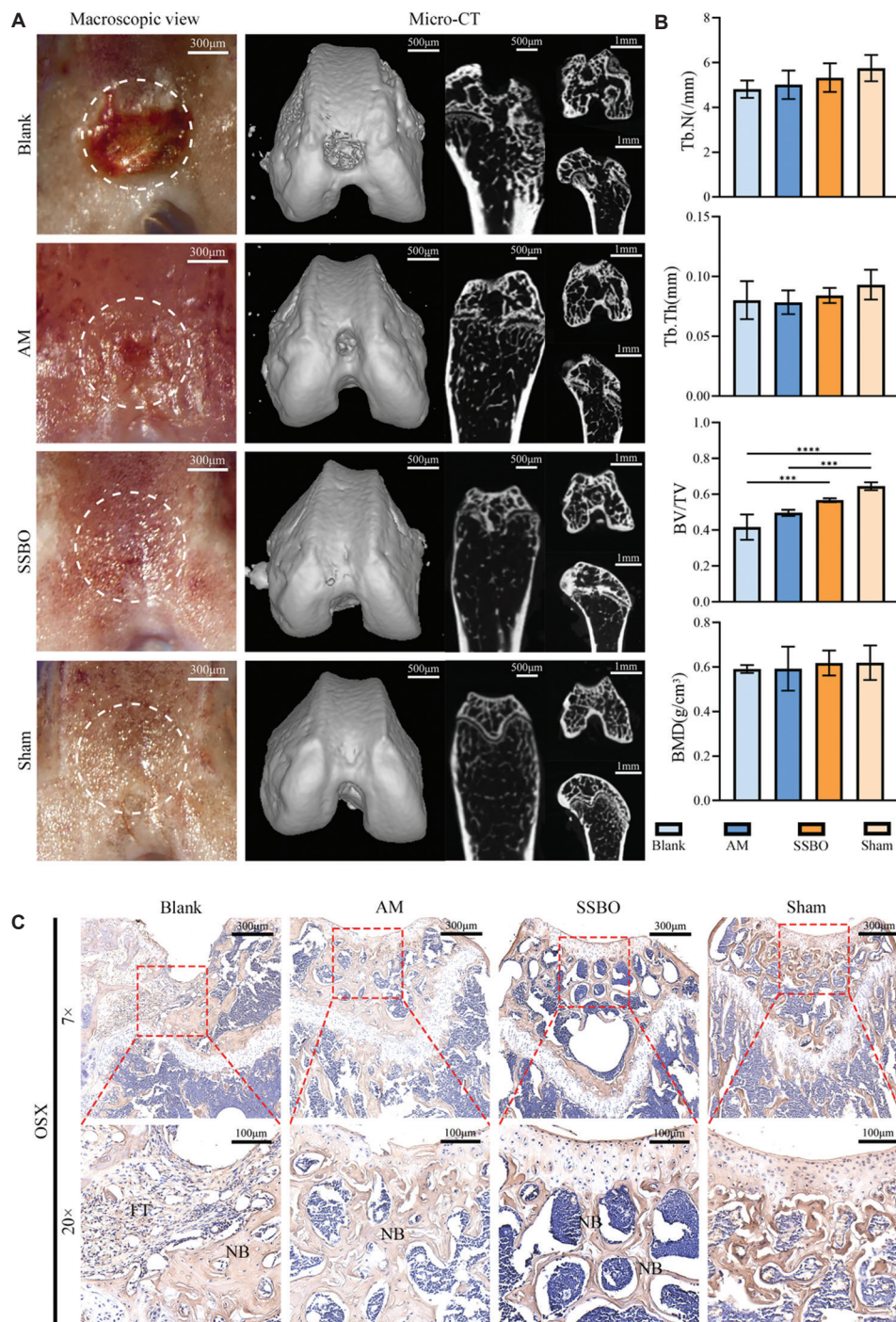


Figure 7. Evaluation of osteochondral regeneration after 4 weeks of implantation. (A) Morphological appearance of osteochondral repair in each group, 4 weeks post-implantation, and micro-CT images of the regenerated tissue, including overall three-dimensional reconstruction, coronal view, transverse (axial) view, and sagittal view. Scale bars: 300 μm, 500 μm, 1 mm. (B) Quantitative analysis based on micro-CT, showing Tb.N, Tb.Th, BV/TV, and BMD. (C) Immunohistochemical staining for OSX in regenerated bone tissue. Scale bars: 100 μm, 300 μm; magnifications: 20×, 7×.

Notes: Data are presented as mean ± standard deviation ($n = 4$); *** $p < 0.001$; **** $p < 0.0001$.

Abbreviations: AM: Control group (adipose-derived stem cells with cartilage extracellular matrix); BMD: Bone mineral density; BV/TV: Bone volume to total volume ratio; CT: Computed tomography; FT: Fibrous tissue; NB: New bone; OSX: Osterix; RC: Regenerated cartilage; SSBO: Subchondral bone organoid; Tb.N: Trabecular number; Tb.Th: Trabecular thickness.

ADSCs and macrophages. SVF-derived cells not only adhered to the CECM surface but also infiltrated deeply

into the scaffold, accompanied by localized degradation and collagen deposition. ADSCs are known to secrete

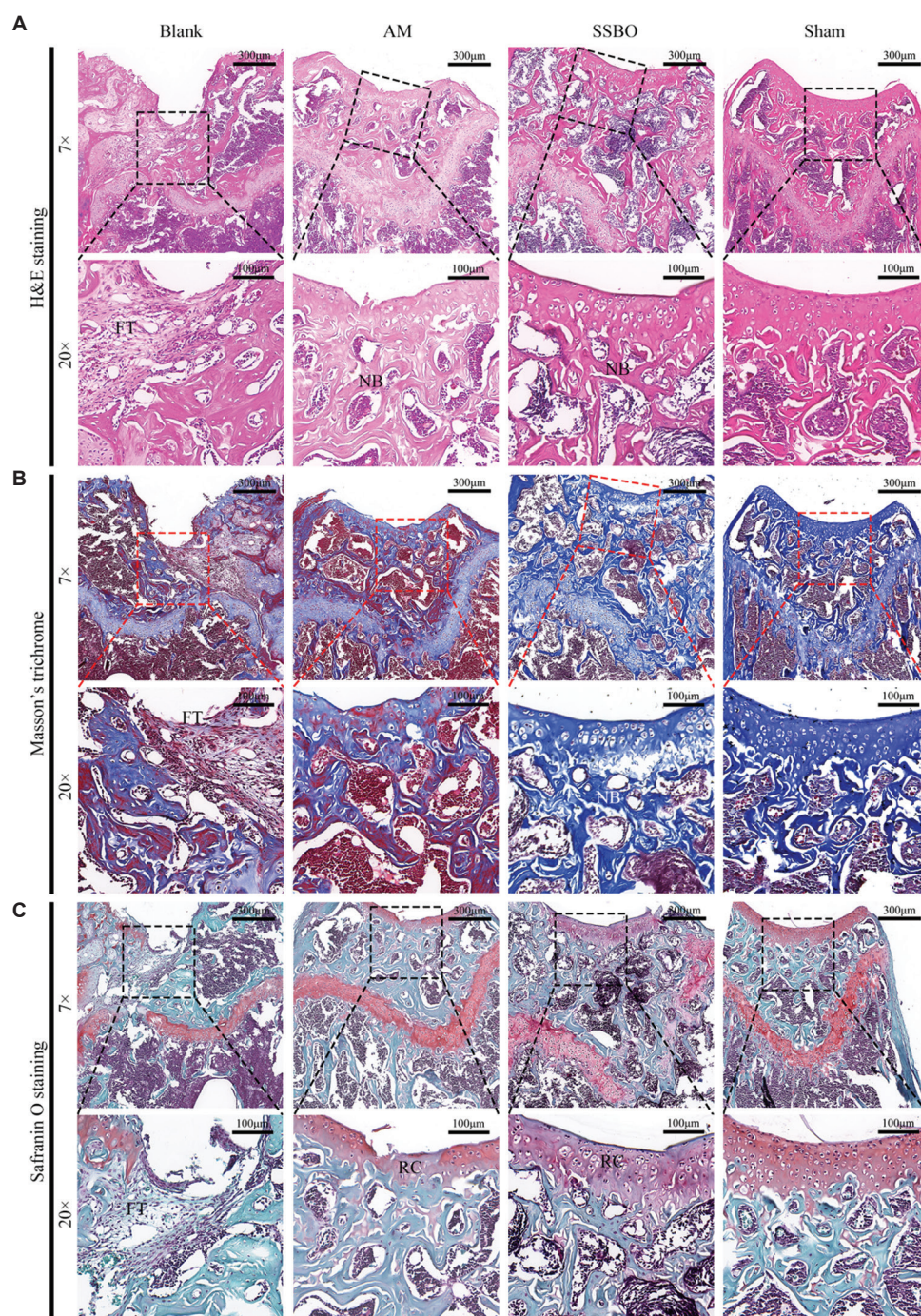


Figure 8. Histological staining at 4 weeks post-implantation. (A) H&E staining of regenerated tissues. (B) Masson's trichrome staining. (C) Safranin O staining of the implanted constructs at 4 weeks post-implantation. Scale bars: 100 μ m, 300 μ m; magnifications: 20 $\times$, 7 $\times$.

Abbreviations: AM: Control group (adipose-derived stem cells with cartilage extracellular matrix); FT: Fibrous tissue; H&E: Hematoxylin and eosin; NB: New bone; RC: Regenerated cartilage; SSBO: Subchondral bone organoid.

matrix metalloproteinases, which initiate the enzymatic breakdown of the ECM.^{42,43} Meanwhile, infiltrating CD68⁺ macrophages may facilitate debris clearance and immune modulation by releasing cytokines and undergoing phenotype switching.⁴⁴⁻⁴⁶ Transmission electron microscopy revealed abundant mitochondria and endoplasmic

reticulum in SSBO cells, indicating enhanced metabolic and biosynthetic activity, in contrast to stress-associated lysosomal features observed in AM controls. Based on these findings, we propose a three-step remodeling model: enzymatic degradation by ADSCs, immune clearance by macrophages, and collaborative matrix reconstruction.

Importantly, ADSCs can also secrete immunomodulatory factors, such as IL-6, prostaglandin E2, and transforming growth factor- β , to promote macrophage polarization toward the M2 phenotype.^{47,48} We found macrophages in the SSBO around the edge of the organoid-like areas. They showed elevated IL-10 levels, suggesting an anti-inflammatory state. IL-1 β levels rose first and then fell. The IL-10/IL-1 β ratio peaked on day 7 and was much higher than in the control group. This suggests that macrophages may shift toward an M2-like state, helping balance the immune system and supporting repair. However, our study only used indirect markers to identify M2-like macrophages. Direct markers, such as CD206 and Arg1, were not tested. Future work should include a broader set of markers using immunostaining or gene analysis. Cytokine profiling and functional tests are also needed. These steps will help define the exact role of macrophages in tissue repair and reveal how they interact with ADSCs.

A hallmark of SSBO is its intrinsic ability to form vascular-like networks *in vitro*, independent of exogenous angiogenic stimulation. Multiplex immunostaining revealed that CD31⁺ EPCs aligned along scaffold channels, forming structured tubular networks by day 14. These were accompanied by PDGFR β ⁺ pericytes, which did not co-localize with endothelial cells but were positioned adjacent to them, suggesting vessel stabilization through paracrine signaling. Interestingly, we observed a mismatch between VEGF and CD31 expression at the mRNA and protein levels. This may result from post-transcriptional regulation, differences in protein stability, or spatial heterogeneity within the organoid. These findings reflect the complexity of self-organization and local regulation in organoid systems. In contrast, AM organoids showed limited spatial organization and lacked vascular markers. The vascularization observed in SSBO is likely orchestrated by the synergistic actions of SVF-derived cell types: EPCs initiate vessel formation, ADSCs enhance angiogenesis through VEGF and basic FGF secretion, and M2-like macrophages promote maturation and ECM remodeling. Pericytes may further stabilize nascent vessels via PDGF and angiopoietin signaling.⁴⁷⁻⁵⁰ This multicellular coordination leads to spatially organized, functional vasculature that supports both perfusion and structural development of the organoid.

Importantly, SSBO also exhibited characteristics of vascular-osteogenic coupling, closely resembling the physiological process of endochondral ossification. Gene and protein expression analyses demonstrated sequential activation of chondrogenic (SOX9, COL2A1), hypertrophic (COL10A1), and osteogenic (RUNX2, COL1A1, OCN) markers. Notably, COL10A1⁺ hypertrophic zones were spatially adjacent to CD31⁺ vascular structures, suggesting synchronized cartilage maturation and angiogenesis. This coupling likely enhances nutrient exchange, cell

differentiation, and matrix remodeling, thus improving scaffold integration and tissue maturation. *In vivo*, SSBO constructs formed chimeric human-mouse vasculature and exhibited superior osteogenic marker expression and bone-like matrix deposition compared to controls. In a mouse osteochondral defect model, SSBO facilitated the simultaneous regeneration of cartilage and SCB, resulting in near-native tissue architecture. These findings underscore the importance of multicellular interactions in orchestrating vascular, immune, and osteogenic processes essential for effective osteochondral repair.

Despite the promising results, several limitations should be acknowledged. First, as a natural biomaterial, CECM may exhibit batch-to-batch variability, necessitating standardized quality control measures for future applications. Second, although SVF contains multiple functional subpopulations, their specific roles in tissue reconstruction have not been directly confirmed by lineage tracing or functional blockade studies. Third, transcriptomic and proteomic analyses were not performed, limiting our understanding of the molecular mechanisms underlying SSBO self-organization. Finally, *in vivo* evaluations were conducted in immunodeficient mice; further validation in large-animal models is required to assess clinical translatability.

5. Conclusion

This study presents a novel approach for constructing SSBO by combining SVF with CECM scaffolds. The resulting SSBO exhibits self-organization, multi-lineage differentiation, vascular integration, and immune modulation. These organoids not only mimic the spatial and functional complexity of native SCB but also demonstrate significant regenerative potential *in vivo*. This approach holds substantial potential for clinical application. SVF cells are autologous, can be harvested intraoperatively, and do not require additional growth factors or genetic modification. These features make SSBO a simple, efficient, and scalable strategy. Furthermore, the SSBO platform can be applied in osteoimmunology studies, personalized therapies for joint disorders, and disease modeling. It provides a promising avenue for future precision medicine in tissue regeneration.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Jiazhou Wu, Zexian Liu, Aiyuan Wang, Hongyu Jiang, Yun Bai

Formal analysis: Tao Qian, Jiazhou Wu, Zexian Liu, Junli Wang, Biao Ma, Jialiang You

Investigation: Zexian Liu, Aiyuan Wang, Yanbin Wu, Zhengrui Zhou, Cheng Huang

Methodology: Tao Qian, Yanbin Wu, Junli Wang, Hongyu Jiang, Zhengrui Zhou, Endong Luo

Writing-original draft: Tao Qian, Jiazhou Wu, Cheng Huang, Junming Zhang, Dingkai Wang

Writing-review & editing: Tao Qian, Yazhou Li, Ying He, Jiang Peng

Ethics approval and consent to participate

This study involving human participants was approved by the Ethics Committee of the Chinese PLA General Hospital (Approval No. 2023KY088-KS001). Adipose tissue samples were collected from nine patients who underwent liposuction at the Fourth Medical Center of the Chinese PLA General Hospital. All participants provided written informed consent prior to inclusion in the study. None of the participants had a history of metabolic or endocrine disorders, alcohol abuse, smoking, or the use of medications affecting glucose or lipid metabolism. All procedures involving human participants were conducted in accordance with the Declaration of Helsinki and the relevant institutional and national ethical guidelines. All procedures involving animals were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Yuma Bio Co., Ltd. (Beijing, China) under protocol number SYXK20250023.

Consent for publication

Written informed consent was obtained from all participants before their inclusion in the study. Each participant explicitly agreed to the use of their clinical samples for research purposes. No personally identifiable information or images of human subjects are included in the manuscript.

Availability of data

The data that support the findings of this study are available from the corresponding author on reasonable request.

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