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REVIEW ARTICLE

Organoid Culture Development for Skeletal Systems

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Organoids are widely considered to be ideal *in vitro* models that have been widely applied in many fields, including regenerative medicine, disease research and drug screening. It is distinguished from other three-dimensional *in vitro* culture model systems by self-organization and sustainability in long-term culture. The three core components of organoid culture are cells, exogenous factors, and culture matrix. Due to the complexity of bone tissue, and heterogeneity of osteogenic stem/progenitor cells, it is challenging to construct organoids for modeling skeletal systems. In this study, we examine current progress in the development of skeletal system organoid culture systems and analyze the current research status of skeletal stem cells, their microenvironmental factors, and various potential organoid culture matrix candidates to provide cues for future research trajectory in this field.

Keywords: organoids, 3D model systems, skeletal stem cells, pluripotent stem cells, skeletal systems

Impact Statement

The emergence of organoids has brought new opportunities for the development of many biomedical fields. The bone organoid field still has much room for exploration. This review discusses the characteristics distinguishing organoids from other three-dimensional model systems and examines current progress in the organoid production of skeletal systems. In addition, based on core elements of organoid cultures, three main problems that need to be solved in bone organoid generation are further analyzed. These include the heterogeneity of skeletal stem cells, their microenvironmental factors, and potential organoid culture matrix candidates. This information provides direction for the future research of bone organoids.

Introduction

Neither animal models nor two-dimensional (2D) cell cultures can fully recapitulate the unique 3D microenvironmental niche of human cells, cell-cell interactions *in vivo*, organ-level functions, and complex structure of the human body. Studies have shown that after being cultured *in vitro*, cells will quickly lose their *in vivo* characteristics, with significant changes to their surface markers and physiological properties.[1] Stem cells also tend to lose their stemness and undergo senescence over prolonged durations of *in vitro* 2D culture.[2–4] On the other hand, because of vast discrepancies between animal models and human physiology, it is often difficult to extrapolate the molecular mechanisms identified in animal models to humans. Biomedical researchers have been attempting to recapitulate the overall niche of normal biology *in vitro* and emphasize simplicity of experiments and availability of cell lines. For

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these purposes, organoid culture systems have thus been developed.[5]

The advent of human induced pluripotent stem cell (iPSC) technology and the availability of a variety of human adult stem cells (ASCs) have created conditions for the emergence of tissue- and organ-specific *in vitro* models.[6] Organoids are well-organized long-term 3D cultures that are formed by stem cells (PSCs or ASCs) through self-organization, which have the capacity to differentiate into some or all specific cell types of the original tissue and can recapitulate structures and at least some functions of the original tissues/organs.[7–9] Indeed, natural human tissues would be attractive substitutes for animal models. Organoids complement existing model systems and provide insight into physiological human development and disease pathogenesis.

Compared to soft tissues/organs such as the intestines, stomach, and brain, the bone has unique characteristics. It contains both hard mineralized bone and soft bone marrow, which means that the stiffness requirements of cells are heterogeneous, and researchers may need to build a complex environment with at least two varying levels of stiffness at the same time. In recent years, researchers have developed some organoid models of skeletal systems, including bone marrow organoids, cartilage organoids, trabecular bone organoids, and others. The appearance of these 3D model systems has provided guiding cues for further development of skeletal system organoids.

This review will therefore analyze the research status of skeletal system organoids, and attempt to identify future research directions in this field by analyzing the current research status of skeletal stem cells (SSCs), their microenvironmental factors, and various potential organoid culture matrix candidates. We emphasize the self-assembly characteristics of organoids, and the construction of an appropriate *in vitro* environment to simulate the *in vivo* stem cell niche, so as to maintain the self-renewal capacity and multilineage differentiation potential of stem cells within organoids.

Organoids Are Good *In Vitro* Culture Model Systems

The earliest use of the term ''organoid'' can be traced back to 1946, when Smith and Cochrane referred to cases of cystic teratoma as ''cystic organoid teratoma.''[10] In 2008, 3D cerebral cortex tissues were constructed from embryonic stem cells (ESCs) using a technique involving serum-free culture of embryoid body-like aggregates (SFEBq culture).[11] The pioneering work of the Clevers research group in constructing continuously expanding intestinal organoids is recognized as a major milestone in the organoid research field.[12,13] This study showed that stem cells can maintain their self-renewal capacity and produce stable and physiologically functioning epithelial cells upon treatment with a specific panel of growth factors.

A variety of different biological materials can be used to form organoids, including ESCs, iPSCs, ASCs, and tissue fragments. Organoids can be categorized according to the initiating cell types that were originally utilized for their production. One of these are the organoids produced by PSCs, that is, organoids generated either by ESCs from cell clusters in blastocysts or iPSCs derived from somatic cells. The other is organoids produced by ASCs, that is, adult stem cells or isolated primary tissue fragments that can self-organize and form 3D cultures *in vitro*.[5] ASC-derived organoids are considered to simulate the process of tissue repair, while PSC-derived organoids model tissue development.[14–16]

Initially, constructed organoids were only composed of epithelium, and the lack of communication between organs and tissues was considered to be one of the defects of existing organoid systems. In recent years, scientists have been capable of integrating mesoderm and even the three germ layers into PSC-derived organoids.[17–20] ASC-derived organoids could also be assembled from endothelial cells and fibroblasts to form more complex structures[21] (Fig. 1). Assembly can also occur between organoids, and the culture was named as assembloids.[22]

Although the field of organoid cultures still has many challenges to be addressed, including limited maturity and size, lack of vascularization, and inability to recapitulate communication between organs, it is still a good model system for regenerative medicine research.[23,24] Organoid opens up a new avenue for pathogenesis research and personalized drug screening for treatment of infectious diseases,[25,26] genetic diseases,[27,28] and cancer.[29,30] In addition to drug screening and molecular medicine,[25,29] the potential value of organoid systems in basic biological research should not be understated.[31] Studies on optic cup organoids have revealed the morphogenetic mechanisms of optic cups and their species specificity.[32,33] In addition, organoids have demonstrated their potential as a minimalist model system to study the principle of self-organization.[34] *In silico* mathematical modeling has also been used to explore the molecular mechanism of organoid self-organization and predict the impact of various microenvironmental conditions on culture results.[35,36]

Organoid Systems Are Distinguished by Sustainability in Long-Term Culture and Capacity for Self-Organization

Organoid technology debuted more than 10 years ago, and since then, various organoid culture systems have been developed. In addition to extracellular matrix (ECM) scaffolds, organoid suspension culture systems, rotating bioreactor, air-liquid interface (ALI) method, and other methods can also be used to produce organoids.[13] No matter what method is used, to label any 3D culture as an organoid system, the following two core characteristics must be present:

First, stem cells are able to self-renew and mature into terminally differentiated cells under artificial *in vitro* culture conditions. The self-renewal capacity allows organoids to be cultured for up to more than a year, which contain proliferating cells throughout the entire culture period, while pluripotency allows stem cells to differentiate and produce different cell lineages, similar to normal tissues.[37] It must, however, be noted that by definition, the self-renewal property should not be attributed to organoids, but should instead be assigned to stem cells. In the organoid passage process, an organoid is dissociated into multiple fragments. Some fragments contain stem cells, which then form multiple organoids, creating the illusion of organoid self-renewal. Rationally, the ability of organoids to replicate themselves and their sustainability in long-term culture are

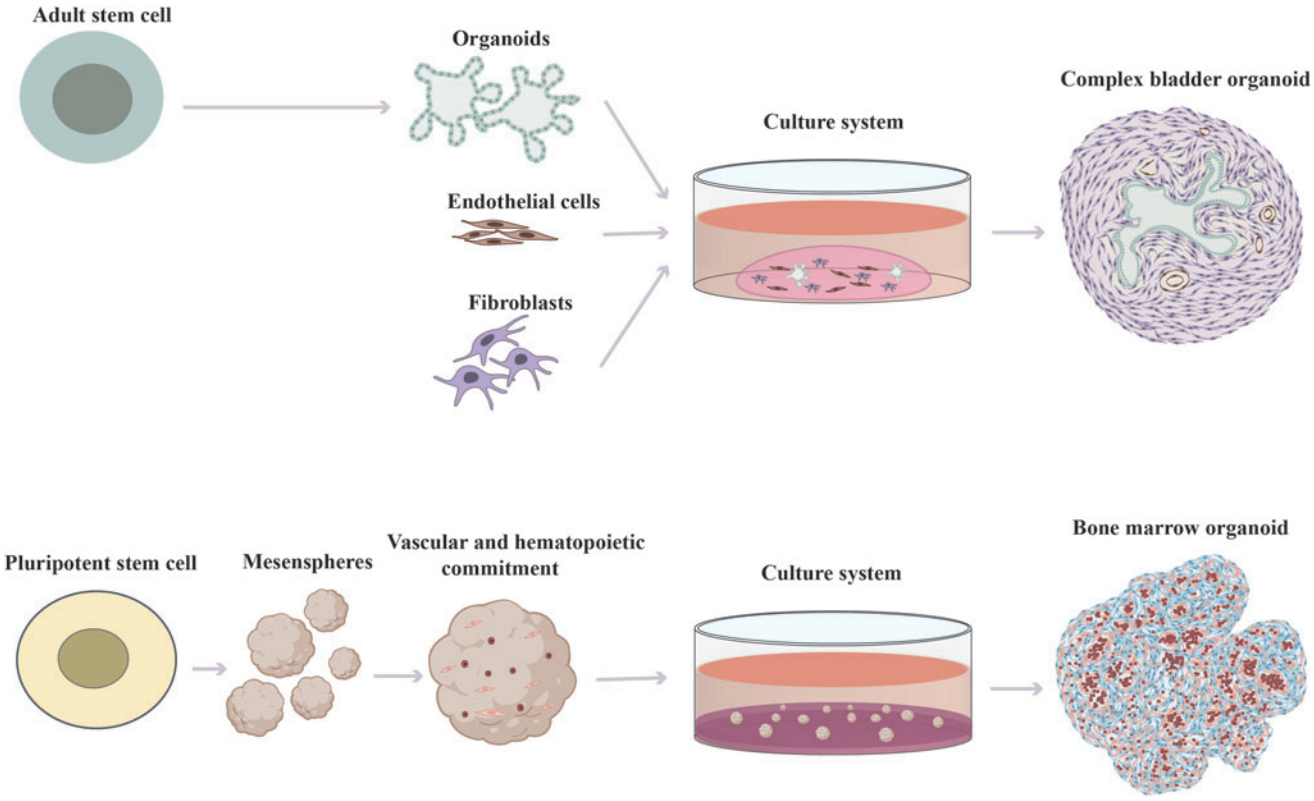

FIG. 1. Schematic illustration of constructing complex organoids with adult stem cells and pluripotent stem cells, taking the bladder and bone marrow as examples, respectively. Color images are available online.

due to the self-renewal capacity of stem cells existing within organoid fragments.

Second, the other key distinctive trait is that organoids are self-organized/self-assembled and highly ordered,[9] recapitulating cellular self-organization during the process of organ formation *in vivo*.[38] This is one of the most important distinctive characteristics that differentiate organoids from ''spheres'' and other 3D models. In some other 3D culture systems such as organs-on-a-chip, cells are induced to differentiate before seeding on specific regions, without higher-level cell sorting or sequencing.[39,40]

Other 3D model systems, including scaffold-free self-organizing spheres, scaffold-based tissue engineering methods, 3D printing products, microfluidics, and organ-on-a-chip, also strive to accurately simulate the physiological state of tissues and the physiological behavior of cells. However, careful evaluation should be conducted before labeling these as organoids.

Organoid Culture for Skeletal Systems

Over the years, researchers have developed *in vitro* and *in vivo* models of bone formation with varying levels of complexity, to simulate the physiological conditions of natural bone,[41–43] cartilage,[44] or bone marrow[45] with complete functions. The appearance of these 3D models provides cues for the construction of skeletal system organoids and helps shape this field. However, as explained above, organoids are *in vitro* 3D model systems, but the reverse statement does not apply. In this review, 3D bone formation models that were labeled as organoid, including bone organoids, bone marrow organoids, osteochondral organoids, cartilaginous organoids, and callus organoid, were carefully evaluated and collectively referred to as skeletal system organoids.

Since 2003, bone marrow stem cell (BMSC) precipitates obtained by centrifugation were induced to form cartilage and subsequently undergo mineralization, with the derived culture being known as ''chondro-osseous organoid.''[46] Pievani et al. used ''heterotopic bone/marrow organoid'' to refer to ossicles formed by BMSCs transplanted *in vivo*.[47] In the past decade, various skeletal system organoids with varying levels of complexity have been reported.

Abraham et al. harvested cell isolates from bone, cartilage, and rib tissues and mixed them with endothelial cells to construct bone, cartilage, and bone-cartilage organoids. The product could be sustainably cultured *in vitro* for 4 months.[48] Similarly, primary human BMSCs were inoculated on porous 3D silk fibroin scaffolds and cultured in a spinner-flask bioreactor to obtain woven bone organoids.[49] In addition, Blache et al. co-cultured mesenchymal stem cells (MSCs) with endothelial cells in round-bottom poly(ethylene glycol) (PEG) hydrogel microwells to produce bone marrow organoids with *in vivo* functional characteristics.[50] Hematopoietic stem/progenitor cells could be recruited and localized around the endothelial network. Although the long-term maintenance of bone marrow organoids was not verified, the self-renewal capacity of the mesenchymal compartment was indicated by its immunophenotype maintenance.

Human periosteum-derived cells (hPDCs) were also reported to follow the early pattern of endochondral ossification

and spontaneously assemble *in vitro* into large-scale bioengineered tissues. This promoted healing of critical sized long bone defects after transplantation in mice. However, in this culture, the number of proliferating cells almost disappeared after 4 weeks of culture.[51]

Park et al. developed a tissue-engineered bone trabecular model by combining osteoblasts and demineralized cortical bone paper. The product was called trabecular bone organoid and simulated the local bone remodeling process within the trabecular cavity. However, it did not contain osteocytes, and the number of cells and maturation stage might not reflect actual bone tissue physiology.[52]

In addition to using ASCs and osteoblasts, human iPSCs were also reported to generate callus-like organoids. In two independent laboratories, murine iPSCs were first differentiated in adherent cultures to acquire chondrogenic potential and were then cultured as spheres and nodules in suspension culture to obtain osteochondral organoids and cartilaginous organoids, respectively.[53,54] A recent breakthrough in the development of bone marrow organoids was made by Workman et al. They used human iPSCs to produce vascularized human bone marrow organoids.[18] These well-organized organoids simulated key features of human bone marrow, recapitulated the molecular interactions of stromal, endothelium and hematopoietic cells, and not only supported active endogenous hematopoiesis but also the growth and survival of normal hematopoietic cells and malignant cells from adult donors. However, whether this could be sustained in long-term cultures remains unknown.

Table 1 lists various skeletal system organoids fabricated by various techniques, as reported in the scientific literature. Among the skeletal system organoid systems, only a few cultures were constructed by self-assembly of stem cells, while many others were obtained by combination of osteoblasts or stem cells together with processed xenogeneic bone or 3D printed scaffolds, which were much more similar to tissue engineered 3D models (Fig. 2). These studies provided valuable cues and experience for the organoid construction of skeletal systems.

However, the skeletal system organoids reported so far have not clarified some or all the following aspects: first and foremost, stem cells should be able to self-organize without restriction. This is crucial for maintaining their unique phenotype and response to the external environment.[55,56] Second, the plating efficiency and specific cell origin of the generated organoids need to be validated by the observation of organoid formation from a single cell.[57] Third, the specific cellular components in organoids should be well defined and clarified. Last but not least, it should be verified that the generated organoid stem cells can self-renew to establish a sustainable long-term culture system.[37,58]

Indeed, there are many challenges in generating skeletal system organoids. The key to successful organoid cultivation is creating an appropriate culture environment to match the natural niche microenvironment of stem cells, so as to allow them to self-renew and mature into at least some cell types of the original tissue at the same time.[31] However to date, the heterogeneity of skeletal stem/progenitor cells has not yet been fully characterized. Microenvironmental factors that affect these independent heterogeneous groups, such as mouse skeletal stem cells (mSSCs), have been sporadically reported in some articles, but there is still no article that fully summarizes these niche factors. In addition, some well-developed and fully defined synthetic materials have been proposed to replace Matrigel as the 3D matrix of choice for organoid culture. These three aspects are the core elements of organoid construction,[59] and will subsequently be discussed in detail in the next section.

Core Elements of Skeletal System Organoid Cultivation

Initiating cell types

When developing organoid systems, especially those derived from ASCs, particular attention must be given to their lineage origin. Researchers have used Cre recombinase technology to track the behavior of stem cells during development and have observed how a single cell forms an entire organoid to prove this point.[12,58] The interbatch variability, heterogeneity, and ambiguous origin of BMSCs pose major challenges that hinder the study of skeletal system organoids.[60] Stem cells are specialized self-renewing and multipotent cell types that contribute to turnover of tissue ontogeny, growth, and lifelong regeneration. Figure 3 shows the currently widely recognized murine osteogenic stem cells, including BMSCs and SSCs, and illustrates their main distribution areas within the bone (Fig. 3).

BMSCs. BMSCs are nonhematopoietic ASCs in bone marrow and can differentiate into various tissues such as bone, fat, and cartilage *in vivo* and *in vitro*. It is confirmed that several murine BMSC subtypes have the capacity to self-renew during *in vivo* sequential transplantation and undergo trilineage differentiation.[61–64] These cell types include PαS cells (cells express PDGFRα and Sca-1),[61] Nestin-expressing cells,[62] and cells expressing leptin receptor (LepR).[64]

These are perivascular cells around arterioles or sinuses. Of note, the cell population isolated by a single marker is heterogeneous. Both $Nestin^+$ cells and $LepR^+$ cells can be broadly categorized into two groups, one predominantly located around arterioles and another subpopulation that is mainly associated with sinusoids.[65–67] Moreover, these markers are found to be nonspecifically expressed by other cells, including chondrocytes, endothelial cells, pericytes, and bone marrow-derived endothelial cells.[68–70] Overlap between PαS cells, leptin receptor-positive ($LepR^+$) cells, and $Nestin^+$ cells has been reported by a number of previous studies,[63,65,71] which led to some controversy about the importance and location of these cell types.

The recent application of single-cell RNA sequencing to investigate bone marrow stroma composition unveils the *bona fide* identity of murine MSCs *in vivo*.[66–68,72] Single-cell analysis of murine whole bone marrow cells conducted by Baccin et al. placed Ng2- and Nestin-expressing mesenchymal cells at the apex of a differentiation level, while cell populations that had transcriptomic similarities to $LepR^+$ cells, osteoblasts, chondrocytes, and fibroblasts were placed downstream.[67] However, it is inappropriate to draw any conclusion based only on the results of single-cell sequencing, and the data need to be further verified by further research.

With regard to human BMSCs, a subendothelial cell population expressing melanoma-associated cell adhesion

Table 1. Skeletal System Organoids and Their Fabrication Protocols

Fabrication schemes	*Noteworthy details*	*Materials*	*Cell source*	*Adherent culture carried out before 3D culture*	*Long-term expansion*	*Culture products*	*Reference*
Suspension culture	Cells were centrifuged to generate pellets	No	Human BMSCs from iliac crest marrow aspirates	20–25 days in adherent culture	Not mentioned	Chondro-osseous organoids	46
	Cells were seeded in round-bottom microwells	PEG hydrogel microwells	Purchased human MSCs and endothelial cells	No	Not mentioned	Human bone marrow organoids	50
	Agarose microwell inserts were used for microspheroid formation	Agarose microwell inserts	hPDCs	hPDCs were expanded until passage 7 to 10	Proliferating cells were almost absent after culturing for 4 weeks	Callus organoid	51
	Cells formed spheroids by centrifugation	No	Murine iPSCs	Cells differentiate to chondrogenic lineage	Not mentioned	Osteochondral organoids	53
	Cartilage nodules were formed by cell differentiation in adherent culture	No	Human iPSCs	Cells differentiate for 14 days	Not mentioned	Cartilaginous organoids	54
Using hydrogel scaffold	Hydrogels containing BMSCs were implanted subcutaneously to form organoid	Hybrid TG-PEG/HA hydrogels	Unsorted human BMSCs from bone marrow aspirates	Not mentioned	Not mentioned	Bone marrow organoid	103
	Cells were seeded on porous 3D silk fibroin scaffolds and cultured within a spinning bioreactor	Silk fibroin scaffolds	Unsorted human BMSCs isolated from bone marrow	Cells were cultured to passage 4	Not mentioned	Woven bone organoids	49
	Cells were resuspended in Matrigel	Matrigel	Mixed MSCs and endothelial cells produced by digestion of human cartilage and bone	No	4 months	Bone organoids, cartilage organoids, and ‘‘mini-joint’’ models	48
	Cells were resuspended in Matrigel	Matrigel	Human iPSCs	No	Not mentioned	Human bone marrow organoids	20

(*continued*)

Table 1. (Continued)

Fabrication schemes	*Noteworthy details*	*Materials*	*Cell source*	*Adherent culture carried out before 3D culture*	*Long-term expansion*	*Culture products*	*Reference*
Seeding cells on processed xenogeneic bone	Cells were seeded on demineralized bone paper	Demineralized bone paper	Mouse osteoblasts	Cells were passaged less than five times	Not mentioned	Trabecular bone organoids	52
	Cells were co-cultured with trabeculae in inverted drop culture systems	Femoral head microtrabeculae	Human osteoblast and osteoclast precursors	Osteoblasts were cultured to passage 3–4; osteoclast precursors were used directly	Not mentioned	Trabecular bone organoids	117
3D printing strategies	Cells were cultured in a biomaterial organoid bioreactor based on skeletal muscle tissue	3D printed β-TCP/HA devices	Not mentioned	Not mentioned	Not mentioned	Osteogenic organoid	118
	Cells were seeded on 3D printed scaffolds and cultured in cyclic-loading bioreactors	3D bioprinted human MSC-laden graphene oxide composite scaffolds	Human MSCs	Cells were cultured to passage 3	8 weeks	Bone organoid	119
	Cells were mixed into bioinks and 3D bioprinted	GelMA matrix mixed with calcium silicate nanowires	Rat BMSCs and rat Schwann cells	BMSCs were cultured to passage 2–5; Schwann cells were cultured to passage 4–9	Not mentioned	Neural-bone construct or innervated-bone organoid	120
	3D bio-printed modules were implanted into bone defects to form organoids	Decellularized matrix-modified 3D-printed PCL scaffold	Not mentioned	Not mentioned	Not mentioned	Organoid bone	121

3D, Three dimensional; BMSCs, bone marrow stromal cells; GelMA, gelatin methacryloyl; HA, hyaluronic acid; hPDCs, human periosteum-derived cells; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem cells; PCL, polycaprolactone; PEG, poly(ethylene glycol); β-TCP, beta-tricalcium phosphate; TG, transglutaminase.

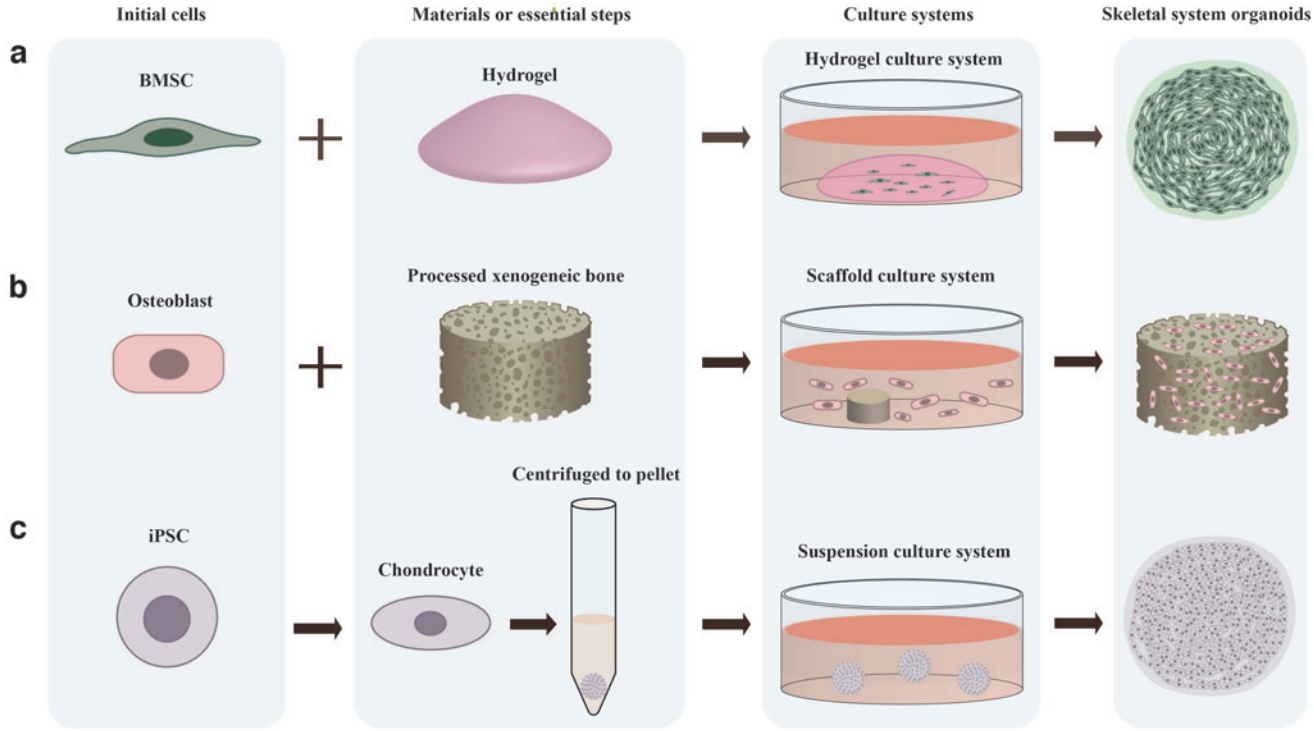

FIG. 2. Examples of skeletal system organoid fabrication systems. **(a)** BMSCs self-assemble in hydrogels; **(b)** osteoblasts combined with processed xenogeneic bone and **(c)** iPSCs are induced to differentiate into chondrocytes, followed by centrifugation to form pellets and finally being cultured in suspension. BMSCs, bone marrow stem cells; iPSCs, induced pluripotent stem cells. Color images are available online.

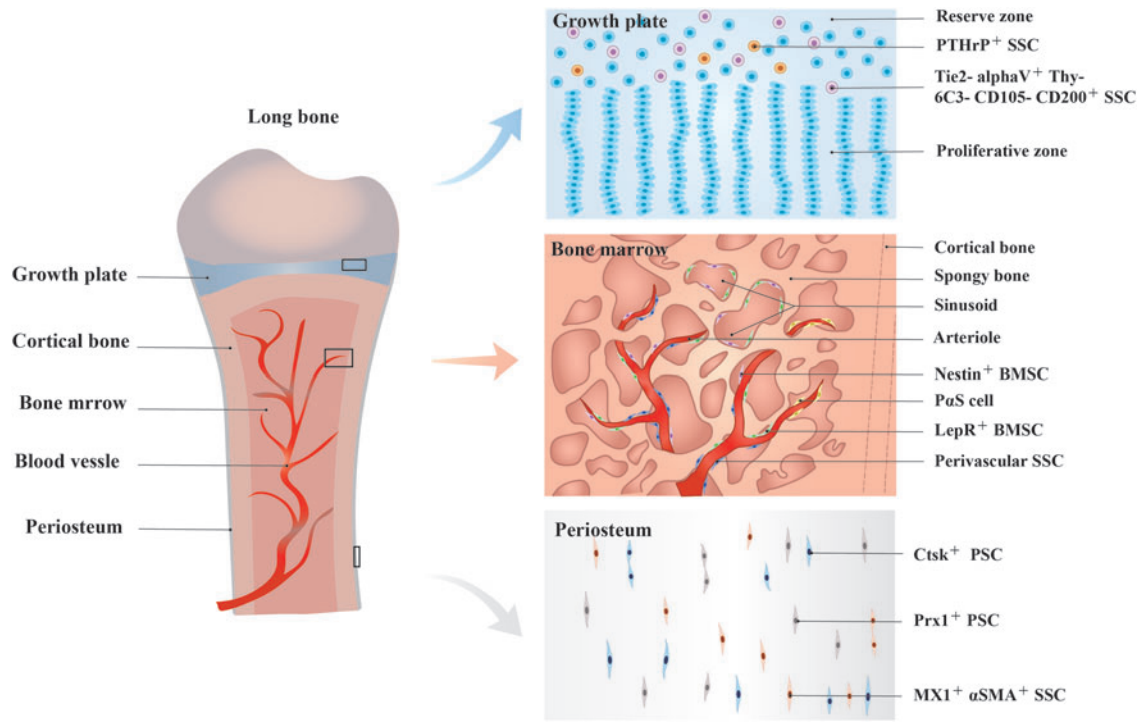

FIG. 3. Currently widely recognized osteogenic stem cell lineages in long bone and their main distribution areas. $PTHrP^+$ SSCs and $Tie2^-$ $alphaV^+$ Thy^- $6C3^-$ $CD105^-$ $CD200^+$ SSCs are mainly distributed in the reserve zone of the growth plate. $Nestin^+$ and $LepR^+$ BMSCs are located around arterioles and sinusoids. $PDGFR\alpha^+$ $Sca\text{-}1^+$ (PαS) cells are localized in the perivascular space near the inner surface of the cortical bone. Perivascular SSCs are localized around blood vessels. $CtsK^+$ and $Prx1^+$ periosteal stem cells and $MX1^+$ αSMA^+ SSC are localized predominantly in the periosteum. CtsK, cathepsin K; LepR, leptin receptor; MX1, MX dynamin like GTPase 1; PTHrP, parathyroid hormone-related protein; αSMA, alpha smooth muscle Actin; SSCs, skeletal stem cells. Color images are available online.

molecule (MCAM/CD146) has been identified in human bone marrow stroma.[73] The generation of heterotopic ossicles within bone marrow and establishment of ectopic hematopoietic microenvironment were demonstrated in that study. CD271 has been identified to be a marker of a subpopulation of self-renewing and PSCs in bone marrow.[74,75] One subpopulation that was specifically labeled by $CD271^+$ $Thy1^+$ $VCAM\text{-}1^{hi+}$ marker combination could home into the bone marrow and maintain their proliferation and multipotency after transplantation *in vivo*. Other studies reported human MSC markers include CD73, CD49a,[76] endoglin (CD105),[77] and mesenchymal stem cell antigen-1.[78]

SSCs. The study of Chan et al. identified various specific and highly purified pluripotent bone progenitor cell populations. The $CD45^-$ Ter^-119^- $Tie2^-$ $alphaV^+$ Thy^- $6C3^-$ $CD105^-$ $CD200^+$ cell subpopulation was identified as multipotent and self-renewing postnatal mSSCs.[79] Further exploration of *bona fide* human skeletal stem cells (hSSCs) revealed that the $PDPN^+$ $CD146^-$ $CD73^+$ $CD164^+$ cell subpopulation exhibited the greatest homology in terms of gene expression to mSSCs, and their self-renewal capacity and pluripotency were validated through elegant continuous transplantation experiments.[80] Both mSSCs and hSSCs are located in the growth plate and can generate progenitor cells of bone, cartilage and stroma, but not fat. In addition, resting chondrocytes that express parathyroid hormone-related protein were identified as a subgroup of SSCs and progenitor cells in the growth plate.[81]

Another group of SSCs were identified around the blood vessels. These were $CD45^-$ $CD31^-$ $PDGFR\alpha^+$ $Sca1^+$ $CD24^+$ cells, which were termed as perivascular SSCs. In addition to differentiating into bone and cartilage, these cells had adipogenic potential and formed bone marrow adipocytes after transplantation *in vivo*.[82] These formed a distinct SSC subpopulation from the primarily identified mSSCs, and did not overlap with each other.[83]

The distribution of SSCs was also found in periosteum and fracture calluses.[84–86] Debnath et al. identified periosteal stem cells that are present in murine long bone and skull.[84] These were specifically labeled with cathepsin K (Ctsk) and located at the apex of the differentiation level. Rigorous continuous transplantation experiments validated the pluripotency and self-renewal capacity of these cells.

One other periosteum SSC subpopulation with high bone regenerative potential was characterized by Prx1 expression.[85] These could mature into osteoblasts, adipocytes, and chondrocyte lineages *in vitro*, and possessed higher *in vivo* regenerative potential than BMSCs. The combination of MX dynamin like GTPase 1 (MX1) and alpha smooth muscle Actin (αSMA) expression can be used to selectively label SSCs. $MX1^+$ αSMA^+ cells are present in the periosteum of adult mice, and had the capacity for colony formation and trilineage differentiation potential *in vitro*. These cells retained the expression of $CD140a^+$ and $CD105^+$ after continuous transplantation into skull defect sites.[86]

Endogenous and exogenous signals

Successful organoid culture lies in knowledge of the stem cell microenvironment and their lineage fate regulation mechanisms. The derivation of organoids depends partly/entirely on endogenous and exogenous signals, which mimic the niche factors encountered by stem cells. Current studies have provided some cues to the factors that influence stem cell lineage fate.

Granulocyte colony-stimulating factor inhibits the proliferation of $Nestin^+$ MSCs and downregulates osteogenic-specific differentiation genes in cells. By contrast, the *in vivo* administration of parathyroid hormone stimulated bone marrow $Nestin^+$ MSCs to proliferate and differentiate into the osteogenic lineage.[62] Phosphatase and tensin homolog regulate the quiescence, maintenance, and differentiation of $LepR^+$ cells. Specifically, it can promote differentiation into osteoblasts and inhibit adipogenic differentiation.[64] Jun-B is a very important factor required by stromal cells to proliferate and differentiate into the osteogenic lineage. It controls BMSC fate during bone development. Knockout of Jun-B had been demonstrated to lead to the decrease of osteoprogenitor cell pools, H-type blood vessels, and arteries in bone, while increasing adipocytes.[72]

Decreased proliferation and increased apoptotic activity in diabetic mice SSCs were reversed upon local delivery of Indian hedgehog.[87] Paracrine factors secreted by Schwann cells are also considered to have potential effects on mSSCs in the mandible.[88] Vascular endothelial growth factor (VEGF) was proven to play a role in bone, cartilage, and stromal progenitor ectopic bone formation by attracting host vascular cell migration and stimulating resting chondrocytes to re-enter the hypertrophic state to restore endochondral ossification.[89,90] Co-delivery of bone morphogenetic protein 2 (BMP2) and VEGF inhibitors have been shown to induce *de novo* cartilage formation.[79] In addition, local and temporary application of BMP2 were found to be able to activate SSCs. Co-delivery of BMP2 and colony-stimulating factor 1 antagonist inhibited bone resorption and restored youthful bone regeneration in aged bones.[91]

It must be noted that the use of scattered information to construct organoid systems is far from sufficient. Single-cell sequencing has created conditions for further analyzing the stem cell microenvironment. Chan et al. have used the Gene Expression Commons analysis platform to identify cells that play key roles in the mSSC niche.[79] They have listed potential signaling pathways affecting bone stem/progenitor cell activity and found that paracrine and autocrine signaling pathways play key roles in the SSC niche, which indicates that SSCs and their downstream differentiated cells can interact with each other to promote skeletal development. However, further exploration of the BMSC/SSC microenvironment and screening and identification of relevant growth factors are still needed to construct skeletal system organoids.

3D culture matrix

It is well known that the self-renewal, proliferation, and differentiation of stem cells are regulated by mechanical properties and biochemical characteristics of the ECM.[92–94] Finding a suitable 3D culture matrix to simulate the stem cell microenvironment is one of the most important aspects of organoid culture. Currently, most organoid culture systems are based on recombinant acellular ECM such as Matrigel. Other materials used for organoid culture include synthetic polymer hydrogels, natural biopolymer matrix, various protein engineering materials, and their combinations.[7]

Matrigel is a gel-like mixture containing ECM components such as laminin, type IV collagen, entactin, and

heparan sulfate proteoglycan, as well as some growth factors like transforming growth factor β and fibroblast growth factor.[95] It provides a complex environment for embedded cells, but there are also uncertainties such as interbatch variability. In addition, the mechanical properties of Matrigel, such as stiffness and elasticity, are almost non-adjustable, which seems to limit its application in organoid culture of skeletal systems, as bone is a calcified hard organ with its Young's modulus reaching 1 GPa or even higher.

Synthetic polymer hydrogels are characterized by biological inertia, defined composition, and adjustable mechanical properties to meet the different requirements for matrix stiffness at different development stages such as expansion and differentiation.[96,97] These make them advantageous when applied to organoid culture systems. Some well-developed synthetic polymer hydrogels have been proposed and worth considering for skeletal system organoid culture. Gelatin methacryloyl-based hydrogels are biocompatible and reproducible. and are stable photo-crosslinked hydrogels with adjustable mechanical properties.[98] These have been applied in cartilage tissue engineering and can facilitate chondrocyte survival and differentiation.[99,100]

Other components such as various minerals and gold nanoparticles can be hybridized to enhance the mechanical strength of hydrogel and have been applied in bone tissue engineering,[101,102] indicating its potential application value in skeletal system organoid cultures. Glorevski et al. constructed a fully defined minimal environment for mouse intestinal organoid culture with PEG hydrogels,[96] indicating the potential application value of synthetic matrix in organoid culture system. Other synthetic hydrogels that have been used for organoid culture include PEG/hyaluronic acid (HA) hybrid hydrogels,[103] Amikagel,[104] PEG with four thiol-reactive maleimides,[105] and cell foam.[106]

Collagen is the most commonly used single-component natural biopolymer matrix. It was proposed to be an excellent candidate for bone formation systems as it mimics the bone composition.[107] Utilizing collagen, many organoid culture systems have been successfully developed, including ALI system,[108] collagen gel ring system,[109] bolstering Lgr5 transformational sandwich culture system,[110] and culture systems similar to Matrigel culture.[111] However to date, there has been no report on the application of collagen in the culture of skeletal system organoid cultures. Other natural biopolymers that have been employed in organoid culture systems include alginate,[112] HA,[113] and fibrin-laminin.[114]

Protein engineering materials are often composed of recombinant proteins carrying specially designed sequences inspired by natural proteins.[115] DiMarco et al. cultured intestinal organoids in recombinant engineered ECM, a hydrogel whose biomechanical and biochemical characteristics can be strategically decoupled and independently adjusted.[116] Although its storage modulus is far lower compared with bone, its stiffness is an order of magnitude higher compared with collagen. The tunability of recombinant protein is basically unlimited, indicating its unlimited potential application value in skeletal system organoid construction.

Conclusions and Future Prospects

Organoids have benefited many biomedical fields as ideal 3D model systems. Stem cell identification, isolation, and obtaining insight into their microenvironmental needs are the basis of organoid culture. Therefore, further understanding of the microenvironmental niche of BMSCs and SSCs is required for the establishment of skeletal system organoids. Identification of SSCs and analysis of BMSCs heterogeneity have brought much improvements to organoid generation of skeletal systems, but further research is still needed to enable better understanding of how SSCs and BMSCs interact with other components within the bone tissue microenvironment.

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Authors' Contributions

J.Q. and Q.G. contributed equally to this work. J.Q.: conceptualization, data curation, writing-original draft preparation, and software. Q.G.: writing-original draft preparation. L.L., X.Z., Y.L., Z.L., and B.C.H.: data curation. P.Z. and Y.Z.: conceptualization, supervision, validation, writing-review and editing, and funding acquisition. All authors have read and agreed to the submitted version of the article.

Disclosure Statement

The authors declare no conflict of interest in this review.

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