Journal of Oral Biology and Craniofacial Research 14 (2024) 540–546

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

# Spheroids and organoids: Their implications for oral and craniofacial tissue/organ regeneration

Hideaki Kagami [a,*], Xianqi Li [b,c]

[a] Department of Dentistry and Oral Surgery, Aichi Medical University, Aichi, Japan
[b] Department of Oral and Maxillofacial Surgery, School of Dentistry, Matsumoto Dental University, Shiojiri, 399-0781, Japan
[c] Department of Hard Tissue Research, Graduate School of Oral Medicine, Matsumoto Dental University, Shiojiri, 399-0781, Japan

ARTICLE INFO

*Keywords:*
Spheroids
Organoids
Stem cells
Regenerative medicine
Tissue engineering
Oral and craniofacial region

ABSTRACT

Spheroids are spherical aggregates of cells. Normally, most of adherent cells cannot survive in suspension; however, if they adhere to each other and grow to a certain size, they can survive without attaching to the dish surface. Studies have shown that spheroid formation induces dedifferentiation and improves plasticity, proliferative capability, and differentiation capability. In particular, spontaneous spheroids represent a selective and efficient cultivation technique for somatic stem cells. Organoids are considered mini-organs composed of multiple types of cells with extracellular matrices that are maintained in three-dimensional culture. Although their culture environment is similar to that of spheroids, organoids consist of differentiated cells with fundamental tissue/organ structures similar to those of native organs. Organoids have been used for drug development, disease models, and basic biological studies. Spheroid culture has been reported for various cell types in the oral and craniofacial regions, including salivary gland epithelial cells, periodontal ligament cells, dental pulp stem cells, and oral mucosa-derived cells. For broader clinical application, it is crucial to identify treatment targets that can leverage the superior stemness of spheroids. Organoids have been developed from various organs, including taste buds, oral mucosa, teeth, and salivary glands, for basic biological studies and also with the goal to replace damaged or defective organs. The development of novel immune-tolerant cell sources is the key to the widespread clinical application of organoids in regenerative medicine. Further efforts to understand the underlying basic mechanisms of spheroids and organoids will lead to the development of safe and efficient next-generation regenerative therapies.

## 1. Introduction

In recent years, there has been growing interest in spheroids and organoids, leading to a rapid increase in related research. Spheroids are the aggregation of cells into three-dimensional (3D) structures.[1] Spheroid culture is a unique method in which adherent cells are suspended to form cell clusters, as opposed to the conventional flat (two-dimensional, 2D) culture on dishes.[2] Despite the difficulties of adherent cell survival in suspension, spheroid culture has proven to be feasible by allowing cells to adhere to each other and form clumps. Since the early application of this method, which resulted in the successful culture of neural stem cells,[3–5] spheroid culture has gained significant attention as an efficient culture method for somatic stem cells. Organoids are small organ-like tissues that have also received significant attention in recent years.[6] Organoids have the potential to regenerate complex organ structures and recapitulate disease phenotypes to some extent, and have already been applied in drug screening and clinical trial safety evaluations.[7]

In oral and craniofacial research, spheroid cultures have been explored for salivary gland epithelial cells,[8] dental pulp-derived stem cells,[9,10] periodontal ligament-derived stem cells,[11–13] and oral mucosa-derived spheroids.[14,15] Various organoid studies, such as those of the salivary glands, taste bud, and teeth, are underway within the oral and craniofacial regions.[16–25] Despite these advancements, 2D cell culture remains the primary clinical approach, and spheroids and organoids are yet to become the principal players in regenerative medicine.

In this review, we present an overview of the current knowledge regarding spheroids and organoids, and address unresolved issues for future investigations. Additionally, we explore how these culture methods can be harnessed for regenerative medicine in the oral and

* Corresponding author. Department of Dentistry and Oral Surgery, Aichi Medical University, 1-1 Yazakokarimata, Nagakute, Aichi, 4801195, Japan.
*E-mail address:* hideaki.kagami@aichi-med-u.ac.jp (H. Kagami).

https://doi.org/10.1016/j.jobcr.2024.07.002
Received 19 August 2023; Received in revised form 9 June 2024; Accepted 1 July 2024

craniofacial regions by leveraging their unique characteristics. By shedding light on the potentials and challenges, this review will contribute to advancing the use of spheroids and organoids in oral and craniofacial tissue/organ regeneration.

## 2. History of spheroid research

What are spheroids in the first place? How was spheroid culture discovered? There are two different culture methods for cells, i.e., "adherent cell culture" and "floating cell culture (suspension cell culture)." Cells such as lymphocytes circulate in the blood. Since these cells normally float under physiological conditions, they do not adhere to the culture vessel or to each other and can survive and proliferate under floating conditions.[26,27] This is called "floating cell culture." Adherent cell culture is a method for culturing all cell types with adhesive properties, and the cells are grown while adhering to a culture vessel. Because the majority of cells in the body are adherent, this can be referred to as a conventional cell culture method. Most adherent cells cannot survive in suspension and undergo apoptosis. However, certain cell types with stem cell characteristics can survive and proliferate to form spheroids in serum-free environments.[3] Otherwise, by aggregation, adherent cells can be cultured as floating spherical cell clusters without cell death.[28] These are spheroid cultures, and the resulting spherical cell clusters are called "spheroids".[1]

An early and remarkable applications of this method was its success in culturing neural stem cells, which was previously considered impractical.[3–5] When neuron-derived cells were cultured in a serum-free environment with epidermal growth factor (EGF), most of the cells died after 2 days, but the remaining cells underwent cell division.[3] These cells continued to grow and detach from the plate, forming floating spheroidal cell clusters (neurospheres).[29,30] Since its discovery, spheroid culture has gained significant attention as an excellent culture method for somatic stem cells, leading to numerous reports on skin-derived cells,[31,32] mesenchymal stem cells,[33,34] and salivary gland epithelial cells.[8] However, except for mesenchymal stem cells, these spheroids must be formed from cells dissociated from tissues and not from cultured cells. Thus, culture efficiency is relatively low, and the number of studies in the medical and dental fields is limited.

Various spheroid-forming protocols have been continuously developed, primarily in the field of engineering. In addition to well-established floating culture methods, such as droplet culture, in which cells are aggregated by gravity by inverting a cell suspension on the backside of the lid of the container,[35] a method of forming spheroids using a spinner/rotation chamber[36] and the neurosphere protocol, various methods have been developed, including a method utilizing a non-adherent dish with U- or V-shaped microwells,[37] a method using a non-adhesive coating area on the dish bottom,[38] an agarose microwell generated by 3D stamps,[39] and a micro well dish with hydrogel.[40]

Currently, spheroid formation is possible using various methods, and the target cell types are diverse. Although there are some exceptions, most studies agree that spheroid-forming cells exhibit superior stemness with excellent multipotency and differentiation, proliferation, migration, and homing abilities.[2,3,8,13,31]

## 3. What is known and not known about spheroids

Although the characteristics of some types of spheroid-forming cells have been studied in detail,[3,41,42] it remains largely unknown whether there is a common and fundamental mechanism for spheroid formation in various cell types using various methods. To simplify the discussion, we have limited the definition of spheroids addressed in this review, although this does not necessarily mean that cell clusters outside this definition are not spheroids. First, spheroids are thought to be floating spherical cell aggregates that are viable in a suspended state.[28] It might also be necessary to consider differences due to spheroid size. Based on the literature and our own experience, we considered the ideal spheroid size to be 50–250 μm in diameter.[15,43,44] The originally proposed neurosphere appeared to refer only to single cell–derived cell clusters. However, accumulating evidence suggests that spheroids formed from multiple cells also exhibit stem cell characteristics, and it may not be appropriate to exclude them.[2,8] Accordingly, in this review, we simply refer spheroids as "spherical cell clusters with a diameter of about 50–250 μm that can be maintained in a suspended state."

Stem cells are selectively cultured during spheroid formation.[3,8,31] However, the results from our study also showed that the transition from 2D to 3D spheroids quickly changed the cellular phenotype and resulted in a sharp increase in the expression of stem cell markers, suggesting the dedifferentiation of cells into more potent stem cells.[2] What are the fundamental mechanisms that induce these phenotypic changes? One possible explanation for this is the hypoxic environment inside the spheroids. Because there is no blood supply inside the spheroids, oxygen and nutrients to the cells are mediated only by diffusion. Previous studies have shown that the center of spheroids becomes hypoxic,[45] inducing various cellular responses to hypoxia. The role of hypoxia-inducible factors (HIFs) has also been investigated.[46,47] Compared to 2D cell cultures, spheroid-forming cells quickly express higher levels of HIF-2α.[46] Moreover, blocking the HIF signaling pathway suppresses spheroid formation.[47] Hypoxia may activate HIFs, which in turn activates downstream genes involved in stem cell maintenance, cell proliferation, cell migration, and angiogenesis.[48] Although transcriptional response to hypoxic stress is mediated by HIFs, the HIFα subunits are also responsive to other factors including factor inhibiting HIF-1α, sirtuins, and metabolites.[49] Accordingly, HIF may be regulated by mechanisms other than oxygen tension. The HIF signaling pathway may not be the only mechanism regulating spheroid formation and related phenotypic changes. To further enhance the use of spheroids, increased research is needed on the mechanisms that regulate spheroid cell characteristics.

## 4. Mechanical spheroids and spontaneous spheroids

One of the biggest problems in discussing spheroid properties is the various methods of spheroid formation. It is unclear whether spheroids formed under these different conditions are equivalent.

Accordingly, we propose dividing them into two broad categories, i.e., "mechanical spheroids" and "spontaneous spheroids." Cells naturally adhere when in physical contact with each other.[50] "Mechanical spheroids" refer to spheroids that form cell clusters by this mechanism. For example, in the hanging drop method, all the cells in a water drop aggregate and contact each other owing to gravity, forming spheroids.[51] Ideally, this would be a nonselective culture. The basic principle is the same for the stirring method and the method using a U-bottom plate.[52] In contrast, when forming spheroids such as neurospheres, a cell suspension is prepared from tissues (originally non-cultured primary cells), and the only cells that can survive and multiply in a serum-free environment with EGF form spheroids.[3] Because non-stem cells cannot survive under these conditions, this method can be considered as a selective culture of neural stem cells. Since this type of spheroid formation does not require external force and the spheroids are formed spontaneously, we categorized them as "spontaneous spheroids".[2]

To increase the availability of spontaneous spheroids, we developed an easy and efficient method for generating spontaneous spheroids.[2,15,46] When the cells are cultured on a hydrophobic plate with a water contact angle of approximately 90°, stem cells migrate, proliferate, coalesce, and spontaneously form spheroids. Even cells after five passages in a 2D environment can form spheroids; thus, the spheroid-forming efficiency has improved dramatically (Fig. 1).[2] Several essential factors are required for spontaneous spheroid formation, such as bFGF, EGF, and B27.[3,31] In our method, these factors also improve the efficiency but are not essential for forming spheroids,[15] helping to reduce costs. This method enables spontaneous spheroid formation from various cell sources including cell lines (3T3 cells), cortical bone-derived

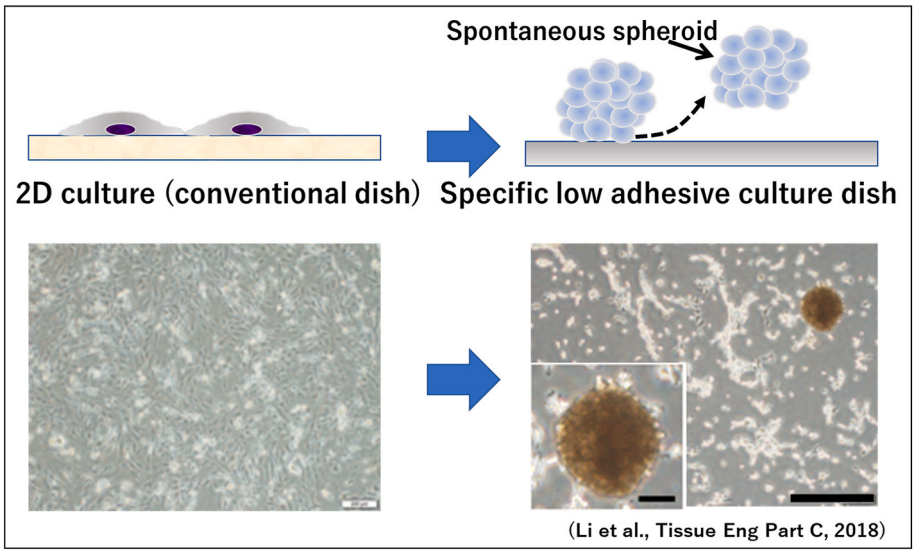


**Fig. 1.** An alternative spontaneous spheroid formation method using a culture dish possessing an almost 90-degree water contact angle. 2D: two dimensional. Modified from Li et al., 2018.[2]

mesenchymal stromal cells, skin-derived cells, and oral mucosa-derived cells.[2,15,46] The obtained spheroids expressed embryonic stem cell markers, such as SSEA-1, SOX2, OCT4, and Nanog, which suggests superior stemness (Fig. 2). Superior plasticity was also confirmed by osteogenic and neural differentiation compared with that of the conventional 2D cultured counterpart (Fig. 3).[15,46,53]

## 5. Potential application of spheroids for oral and craniofacial tissue regeneration

Lombaert et al. succeeded in forming spheroids from salivary gland epithelial cells (salispheres).[8] Their results showed that spheroids were derived from the ductal compartment of the salivary gland, which contains stem cells, and were capable of ameliorating radiation damage when transplanted.[8,42] Moritani et al. focused on stem cells in the periodontal ligament and investigated the effects of spheroid culture.[13] Spheroids formed using microwell chips expressed MSC cell markers, with higher expression levels compared to the cells cultured in a monolayer. Furthermore, periodontal ligament-derived cells cultured as spheroids exhibited enhanced osteogenic differentiation compared to the cells cultured in a monolayer, and upon transplantation, demonstrated superior bone regeneration ability in vivo. We have previously reported that spheroids from cortical bone-derived cells (CBDCs) possess superior osteogenic differentiation capabilities compared to those from 2D-cultured cells.[46] Interestingly, cryopreserved spontaneous spheroids from CBDCs showed remarkable new bone formation in vivo, identical to that of non-cryopreserved spheroids, even without osteogenic induction.[53] A more recent study investigated the bone regeneration capability of jawbone periosteum-derived cells and assembled spheroids using a nonadherent microwells.[54] Compared to monolayer culture, periosteum-derived cell spheroids showed a significant upregulation of chondrogenic markers such as SOX9 and significantly accelerated bone healing when transplanted to a critical size jaw bone defect of mice.

Research on these spheroids targeting salivary glands, periodontal ligaments, and cortical bone-derived cells harnesses the characteristic features of spheroid culture, which enhances stemness compared to monolayer culture and enables efficient selective cultivation of stem cell fractions. However, this approach is not limited to oral and craniofacial tissues. Concurrently, attempts have been made to apply spheroid culture as a means of selectively cultivating neural crest-derived cells from oral and craniofacial tissues. Neural crest-derived cells, originating from the embryonic neural crest, are known to exist in various tissues even in adults.[55] Given their remarkable self-renewal and regenerative capacities, neural crest-derived cells present a highly promising cell source for regenerative medicine. Interestingly, tissues of the oral and craniofacial region, including gingiva and oral mucosa, have been reported to harbor neural crest-derived stem cells.[56–58] Research utilizing spheroid culture to efficiently cultivate and expand neural crest-derived cells from oral and craniofacial tissues can be considered as a unique and promising research leveraging the tissue characteristics of the oral and craniofacial region. Therefore, examples of such studies are also presented herein.

The oral mucosa has also been studied as a target for spheroid culture. Abe et al. reported the formation of spheroids from human oral mucosal cells using a culture method similar to neurospheres.[14] Spheroids obtained from oral mucosa using this method indeed expressed markers of neural crest-derived cells such as nestin, CD44, slug, snail, and MSX1.[14] These cells demonstrated the ability to differentiate into neural, adipogenic, chondrogenic, and osteogenic lineages, suggesting their enrichment with cells maintaining properties of neural crest-derived cells. We also reported that oral mucosal cell-derived spheroids contain highly potent stem cells that can differentiate into neural cells expressing neurogenic markers, such as nestin, βIII tubulin, MAP2, NeuN, Sox2, and S100β.[15] Higher expression of a dopaminergic neuron marker suggests the advantage of using these cells for the treatment of neurodegenerative disorders, such as Parkinson's disease.[15] Since the collection of oral mucosae is relatively easy and minimally invasive, the use of oral mucosal cell-derived spheroids is expected to be applied not only for the regeneration of oral and craniofacial tissue but also in other areas such as neurodegenerative diseases in the future.

## 6. Spheroids and organoids: distinctive properties of 3D cellular aggregates

Spheroids and organoids share similar characteristics with 3D cell cultures. However, they exhibit fundamental differences in their nature. Spheroids are simple clusters of a broad range of cell types,[59] primarily refer to selective stem cell cultures and tend to induce cellular dedifferentiation.[2] In most cases, spheroids are composed of a single cell type and are scaffold-free but occasionally scaffold-dependent.[1] As mentioned earlier, spheroids can be generated by various methods but we designated it into two major categories as "Mechanical spheroids" (methods depend on physical force such as hanging drop method via gravity and rotation culture method using forced cell-to-cell attachment via agitation) and "spontaneous spheroids" (methods based on

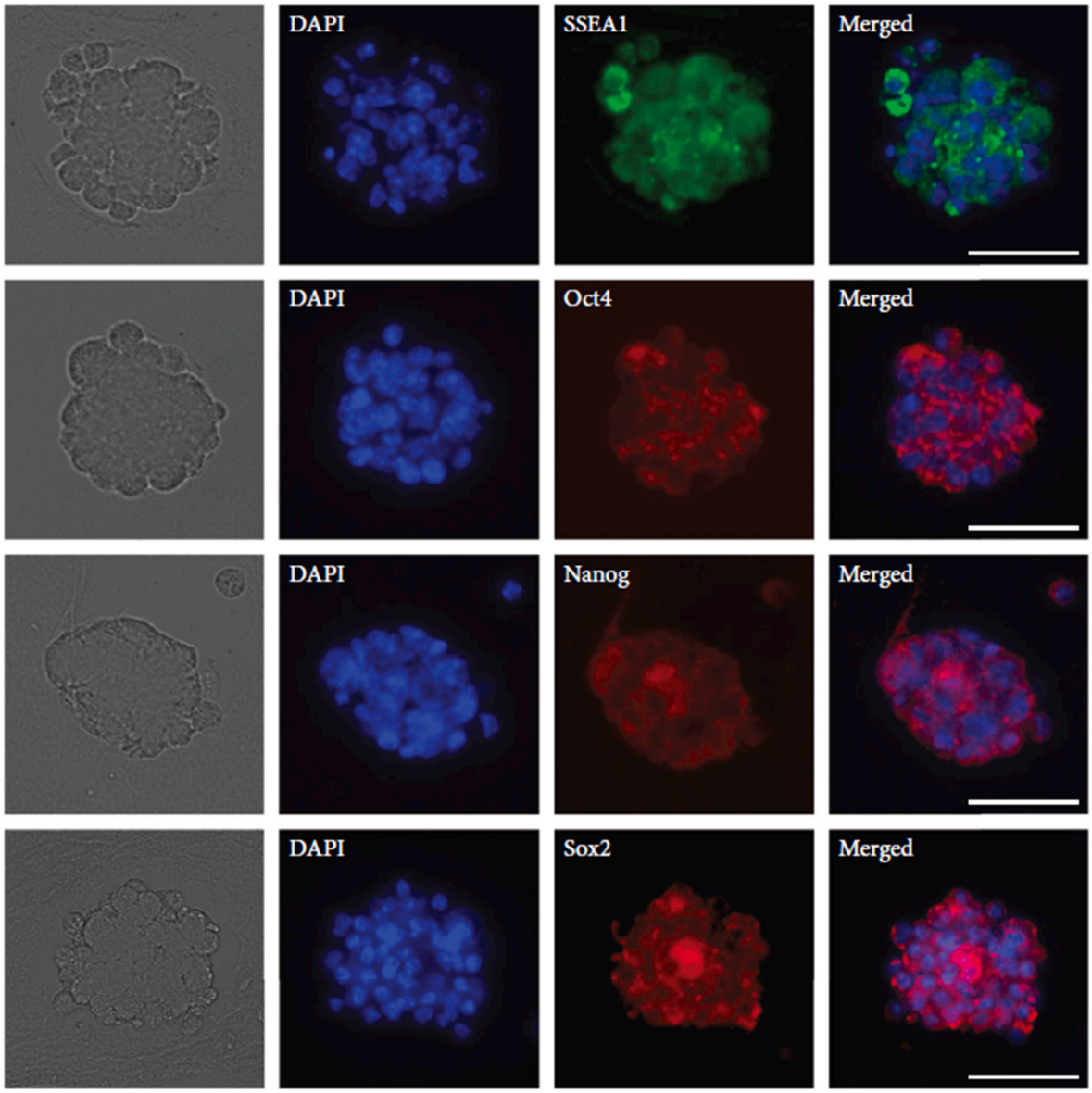


**Fig. 2.** Immunofluorescence staining of spontaneously formed spheroids with compact bone-derived cells (CBDCs). Scale bars = 50 μm. Adapted from Chen et al., 2019.[15]

spontaneous cell growth and aggregation).[2,15] The resultant spheroids serve as valuable materials for tissue regeneration, as the cells within them maintain their original differentiation tendencies and can further differentiate into the desired cell types depending on the transplanted environment.[8,13,53] Other than that, spheroids have been used as multicellular models to test chemical and mechanical interactions and drug response.[59]

Organoids, also known as mini-organs, are three-dimensional cell structures that mimic the functions of tissues and organs of living organisms in vitro. Organoids can be generated from a single type of tissue/organ stem cells but multiple cell types including embryonic stem cells, induced pluripotent stem cells, adult stem cells and endothelial cells are often employed, and sometimes the presence of extracellular matrices is essential.[60,61] The generation of organoid can be achieved by self-organization from a tissue/organ stem cells in a matrix, recapitulation of epithelial-mesenchymal interaction in matrices, or an aggregation of multiple cell types such as endoderm, endothelial, and mesenchymal progenitor populations to form organ-like structure.[61] The organoids mimic complex key structure, function, and biology of organs or tissues from which they are derived.[59] Organoids are intended to use for disease modelling, drug screening and eventually organ replacement (regeneration). Upon transplantation, they are expected to engraft and function as mini-organs, supported by immediate vascular anastomosis and initiation of blood supply from the surrounding tissues.[60] The comparison of spheroids and organoids was summarized in Table 1.

## 7. Organoids: a 3D cellular self-organization for organ mimicry

Organoids are self-organized 3D cell aggregates that recapitulate the morphology and functions of their in vivo counterparts.[6] Compared with 2D cell culture, organoids can effectively simulate gene and protein expression patterns, cell-matrix interactions, and metabolic functions, and have been used for drug screening, as a model for organogenesis and developmental disorders, and tissue/organ regeneration.[6,62]

The discovery of intestinal stem cells is a major research topic in this field. Leucine-rich repeat-containing G-protein-coupled receptor 5-positive (Lgr5+) cells reside at the crypt base.[63] They can generate all intestinal epithelial lineages and are thus considered stem cells of the

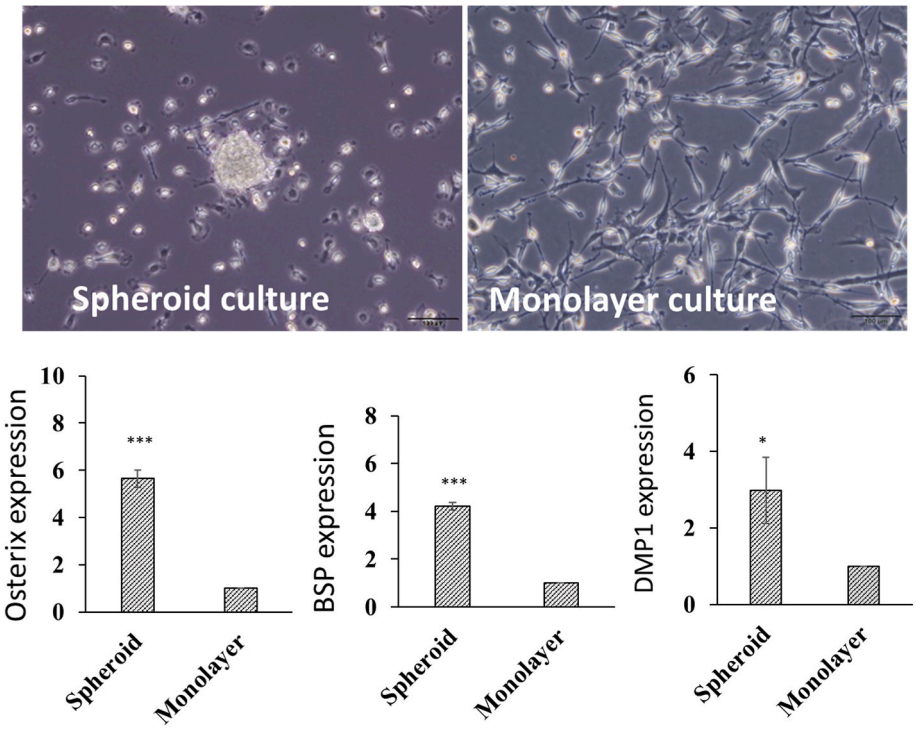


**Fig. 3.** Osteogenic capability of spheroids. Modified from Chen et al., 2019.[15]

**Table 1**
Comparison of spheroids and organoids.

| | Spheroids | Organoids |
|---|---|---|
| Definition | Simple clusters of a broad range of cell types | A self-organized 3D cell structures that mimic the functions of tissues and organs in vitro |
| Cellular composition | Mostly single cell type, occasionally multiple cell types | A single type of tissue/organ stem cells or a mixture of multiple cell types including embryonic stem cells, induced pluripotent stem cells, adult stem cells and endothelial cells |
| Scaffold | Mostly scaffold-free, occasionally scaffold-dependent | Various natural and synthetic scaffolds are used and sometimes the presence of extracellular matrices is essential |
| Method for 3D organization | "Mechanical spheroids" (methods depend on physical force such as hanging drop method via gravity and rotation culture method using forced cell-to-cell attachment via agitation) and "spontaneous spheroids" (methods based on spontaneous cell growth and aggregation). | Self-organization from a tissue/organ stem cells in a matrix, recapitulation of epithelial-mesenchymal interaction in matrices, or an aggregation of multiple cell types such as endoderm, endothelial, and mesenchymal progenitor populations to form organ-like structure |
| Structure | Simple aggregation of cells but primarily refer to selective stem cell cultures and tend to induce cellular dedifferentiation | Mimic complex key structure, function, and biology of organs or tissues from which they are derived |

small intestine and colon, lead to the establishment of intestinal organoids. Another major topic was the establishment of a common organoid production platform for vascularized organoids. Tatebe et al. demonstrated the formation of liver-like structures via mesenchymal cell-driven condensation using induced pluripotent stem (iPS) cells, mesenchymal stem cells, and vascular endothelial cells.[60] and eventually entirely from iPS-derived cells.[64] This method includes not only epithelial cells but also mesenchymal cells, that can compensate for the shortcomings of previous methods. In addition, the organized vasculature enables early blood supply after transplantation. The adaptability of this principle to various organs has triggered an explosive surge in organoid research across various fields.[65,66] Notably, pluripotent iPS cells have demonstrated promising potential for organ regeneration, addressing the most desired clinical needs of regenerative medicine.[64,67,68]

## 8. Applications of organoid technology for oral and craniofacial tissue regeneration

Several intriguing studies have reported the regeneration of oral and craniofacial tissues through the application of organoid technology. Organoid production from a single type of tissue/organ stem cell within the tissue have been demonstrated feasible in some organs. For instance, Lgr5+ cells present in the crypt base are capable of differentiating into all cell types constituting the small intestine, suggesting their role as tissue/organ stem cells.[63] Aihara et al. reported the regeneration of multilayered epithelium, precursor cells, and taste bud cells from Lgr5+ cells within the circumvallate papilla by embedding them in Matrigel, thus demonstrating the regeneration of multilayered epithelium and taste buds similar to the original tissue structure.[17]

Additionally, studies on organ regeneration using organoids composed of multiple cell types are exemplified by research on tooth regeneration. Dental organs are originally formed through interactions between embryonic oral epithelial and mesenchymal cells.[69] While it was initially thought that cell-cell interactions between developing dental organ cells were necessary, it was known that dental organs could form through similar interactions even when using cultured mesenchymal stem cell tissues.[70,71] Reproduction of such interactions within collagen gels has been reported as a method to produce dental organoids.[72] Similarly, research aiming at organ regeneration by mimicking embryonic development is being conducted targeting salivary glands. Tanaka et al. successfully regenerated salivary gland tissues through

organoid cultivation from mouse ES cells by combining two transcription factors, Sox9 and Foxc1.[73] Moreover, they demonstrated the feasibility of regenerating morphologically and functionally normal salivary gland tissues by transplanting regenerated salivary gland organoids into salivary gland tissues.[73] Subsequently, the production of salivary gland organoids using human iPS cells and the regeneration of salivary gland tissues through the transplantation of these organoids have been reported.[74]

However, tissue stem cells capable of regenerating entire organs like Lgr5+ cells in the intestine and taste buds have not been found in tooth germ or salivary glands. Nevertheless, the regeneration of salivary gland tissues from salivary gland organoids produced from ES cells or iPS cells is considered a significant breakthrough towards the clinical application of organoid-based organ regeneration, which are not capable with the simple spheroid cultures. The current limitations of organoids for oral and craniofacial tissues lie in the cell source. For dental organs, developing dental organ-derived cells are necessary in some form, the supply of which is challenging. While cultivation of dental epithelial and dental mesenchymal cells derived from ES cells or iPS cells has been reported,[75] the formation of dental organoids has not yet been achieved. On the other hand, transplantation of iPS cells or ES cells presents issues of immune rejection. It is expected that as these issues are addressed, organoid technology will become more practical.

## 9. How organoids contribute to oral and craniofacial tissue/organ regeneration: prospects and challenges

In recent years, organoid research has garnered significant attention, even in the oral and craniofacial regions. The regeneration of taste buds, oral mucosa, teeth, and salivary glands has been investigated.[17,19–25]

Development of transplantable organs is a highly desirable goal in regenerative medicine. However, conventional tissue engineering and hybrid approaches using artificial materials have not yet succeeded in creating transplantable organs that can maintain long-term functionality comparable to that of transplanted organs. To achieve these goals through organoid research, the following issues need to be solved: the procurement of cell sources that are safe and not subject to immune rejection, the generation of transplantable organoids with therapeutically effective sizes, and the scale-up of organoid production. In particular, the procurement of cell sources suitable for transplantation without needing immunosuppression is of paramount importance. Organoids generated using autologous somatic stem cells, autologous iPS cells, and iPS cells capable of achieving immune tolerance are promising candidates. Advances in research in this area hold promise for the development of transplantable salivary glands and dental buds, and offer new therapeutic opportunities for previously intractable diseases.

## 10. Conclusion

Spheroid culture is an excellent method for culturing stem cells. In particular, spontaneous spheroids represent a selective and efficient cultivation technique for somatic stem cells and should be widely adopted in regenerative medicine as an alternative to conventional 2D culture. Organoid research is rapidly advancing in the oral and craniofacial regions. However, there are still many challenges to be addressed. Organ stem cells that can be harvested from adult tissues and can regenerate all cell types in the original organ have not been found. While organ regeneration has been attempted using ES cells and iPS cells, it has not been successful except for a few organs such as salivary glands. Moreover, these cells still pose issues of immune rejection. Securing safe, immune-rejection-free, and stably suppliable stem cells is likely the primary challenge. There are still no organoids that can be transplanted into humans in oral and craniofacial regions. Achieving early revascularization post-transplantation that can ensure long-term engraftment and scaling up organoids to practical volume are considered the next important challenge. Despite such difficulties, spheroid and organoid technologies are expected to lead to efficient regeneration therapies for severe tissue and organ defects that are difficult to treat using conventional therapies.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## References


1. Lee S-Y, Koo I-S, Hwang HJ, Lee DW. In vitro three-dimensional (3D) cell culture tools for spheroid and organoid models. *SLAS Discov*. 2023;28:119–137. https://doi.org/10.1016/j.slasd.2023.03.006.
2. Li X, Ni Li, Chen Kai, Nagasawa S, Yoshizawa M, Kagami H. Around 90° contact angle of dish surface is a key factor in achieving spontaneous spheroid formation. *Tissue Eng C Methods*. 2018;24:578–584. https://doi.org/10.1089/ten.TEC.2018.0188.
3. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*. 1992;255(5052):1707–1710. https://doi.org/10.1126/science.1553558.
4. Reynolds BA, Tetzlaff W, Weiss S. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J Neurosci*. 1992;12:4565–4574. https://doi.org/10.1523/JNEUROSCI.12-11-04565.1992.
5. Weiss S, Reynolds BA, Vescovi AL, Morshead C, Craig CG, van der Kooy D. Is there a neural stem cell in the mammalian forebrain? *Trends Neurosci*. 1996;19:387–393. https://doi.org/10.1016/s0166-2236(96)10035-7.
6. Yang S, Hu H, Kung H, et al. Organoids: the current status and biomedical applications. eCollection 2023 Jun *MedComm*. 2020;4:e274. https://doi.org/10.1002/mco2.274, 2023.
7. Vandana JJ, Manrique C, Lacko LA, Chen S. Human pluripotent-stem-cell-derived organoids for drug discovery and evaluation. *Cell Stem Cell*. 2023;30:571–591. https://doi.org/10.1016/j.stem.2023.04.011.
8. Lombaert IM, Brunsting JF, Wierenga PK, et al. Rescue of salivary gland function after stem cell transplantation in irradiated glands. *PLoS One*. 2008;3, e2063. https://doi.org/10.1371/journal.pone.0002063.
9. Sasaki R, Aoki S, Yamato M, et al. Neurosphere generation from dental pulp of adult rat incisor. *Eur J Neurosci*. 2008;27:538–548. https://doi.org/10.1111/j.1460-9568.2008.06026.x.
10. Xiao L, Tsutsui T. Characterization of human dental pulp cells-derived spheroids in serum-free medium: stem cells in the core. *J Cell Biochem*. 2013;114:2624–2636. https://doi.org/10.1002/jcb.24610.
11. Yam GH, Teo EP, Setiawan M, et al. Postnatal periodontal ligament as a novel adult stem cell source for regenerative corneal cell therapy. *J Cell Mol Med*. 2018;22:3119–3132. https://doi.org/10.1111/jcmm.13589.
12. Yan XZ, van den Beucken J, Yuan C, Jansen JA, Yang F. Spheroid formation and stemness preservation of human periodontal ligament cells on chitosan films. *Oral Dis*. 2018;24:1083–1092. https://doi.org/10.1111/odi.12855.
13. Moritani Y, Usui M, Sano K, et al. Spheroid culture enhances osteogenic potential of periodontal ligament mesenchymal stem cells. *J Periodontal Res*. 2018;53:870–882. https://doi.org/10.1111/jre.12577.
14. Abe S, Yamaguchi S, Sato Y, Harada K. Sphere-derived multipotent progenitor cells obtained from human oral mucosa are enriched in neural crest cells. *Stem Cell. Transl. Med.* 2016;5:117–128. https://doi.org/10.5966/sctm.2015-0111.
15. Li N, Li X, Chen K, Dong H, Kagami H. Characterization of spontaneous spheroids from oral mucosa-derived cells and their direct comparison with spheroids from skin-derived cells. *Stem Cell Res Ther*. 2019;10:184. https://doi.org/10.1186/s13287-019-1283-0.
16. Nanduri LS, Baanstra M, Faber H, et al. Purification and ex vivo expansion of fully functional salivary gland stem cells. *Stem Cell Rep*. 2014;3:957–964. https://doi.org/10.1016/j.stemcr.2014.09.015.
17. Aihara E, Mahe MM, Schumacher MA, et al. Characterization of stem/progenitor cell cycle using murine circumvallate papilla taste bud organoid. *Sci Rep*. 2015;5, 17185. https://doi.org/10.1038/srep17185.
18. Pringle S, Maimets M, van der Zwaag M, et al. Human salivary gland stem cells functionally restore radiation damaged salivary glands. *Stem Cell*. 2016;34:640–652. https://doi.org/10.1002/stem.2278.
19. Wang F, Li G, Wu Z, et al. Tracking diphyodont development in miniature pigs in vitro and in vivo. *Biol Open*. 2019;8, bio037036. https://doi.org/10.1242/bio.037036.
20. Rosowski J, Bräunig J, Amler AK, Strietzel FP, Lauster R, Rosowski M. Emulating the early phases of human tooth development in vitro. *Sci Rep*. 2019;9:7057. https://doi.org/10.1038/s41598-019-43468-0.
21. Driehuis E, Kolders S, Spelier S, et al. Oral mucosal organoids as a potential platform for personalized cancer therapy. *Cancer Discov*. 2019;9:852–871. https://doi.org/10.1158/2159-8290.CD-18-1522.
22. Sui Y, Zhang S, Li Y, et al. Generation of functional salivary gland tissue from human submandibular gland stem/progenitor cells. *Stem Cell Res Ther*. 2020;11:127. https://doi.org/10.1186/s13287-020-01628-4.
23. Tanaka J, Mishima K. In vitro three-dimensional culture systems of salivary glands. *Pathol Int*. 2020;70:493–501. https://doi.org/10.1111/pin.12947.

24. Xu X, Li Z, Ai X, Tang Y, Yang D, Dou L. Human three-dimensional dental pulp organoid model for toxicity screening of dental materials on dental pulp cells and tissue. *Int Endod J*. 2022;55:79–88. https://doi.org/10.1111/iej.13641.
25. Yoon YJ, Kim D, Tak KY, et al. Salivary gland organoid culture maintains distinct glandular properties of murine and human major salivary glands. *Nat Commun*. 2022;13:3291. https://doi.org/10.1038/s41467-022-30934-z.
26. Oettgen HF, Silber R, Miescher PA, Hirschhorn K. Stimulation of human tonsillar lymphocytes in vitro. *Clin Exp Immunol*. 1966 Jan;1(1):77–84.
27. Neeser A, Ramasubramanian R, Wang C, Ma L. Engineering enhanced chimeric antigen receptor-T cell therapy for solid tumors. *Immunooncol Technol*. 2023;19, 100385. https://doi.org/10.1016/j.iotech.2023.100385. eCollection 2023 Sep.
28. Yen BL, Hsieh C-C, Hsu P-J, Chang C-C, Wang L-T, Yen M-L. Three-dimensional spheroid culture of human mesenchymal stem cells: offering therapeutic advantages and in vitro glimpses of the in vivo state. *Stem Cell. Transl. Med.* 2023;12:235–244. https://doi.org/10.1093/stcltm/szad011.
29. Vescovi AL, Reynolds BA, Fraser DD, Weiss S. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron*. 1993;11:951–966. https://doi.org/10.1016/0896-6273(93)90124-a.
30. Nakamura Y, Sakakibara Si, Miyata T, et al. The bHLH gene hes1 as a repressor of the neuronal commitment of CNS stem cells. *J Neurosci*. 2000;20:283–293. https://doi.org/10.1523/JNEUROSCI.20-01-00283.2000.
31. Toma JG, Akhavan M, Fernandes KJ, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol*. 2001;3:778–784. https://doi.org/10.1038/ncb0901-778.
32. Toma JG, McKenzie IA, Bagli D, Miller FD. Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cell*. 2005;23:727–737. https://doi.org/10.1634/stemcells.2004-0134.
33. Potapova IA, Gaudette GR, Brink PR, et al. Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. *Stem Cell*. 2007;25:1761–1768. https://doi.org/10.1634/stemcells.2007-0022.
34. Wang W, Itaka K, Ohba S, et al. 3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. *Biomaterials*. 2009;30:2705–2715. https://doi.org/10.1016/j.biomaterials.2009.01.030.
35. Dixon AR, Ramirez Y, Haengel K, Barald KF. A drop array culture for patterning adherent mouse embryonic stem cell-derived neurospheres. *J Tissue Eng Regen Med*. 2018;12:e379.
36. Low HP, Savarese TM, Schwartz WJ. Neural precursor cells form rudimentary tissue-like structures in a rotating-wall vessel bioreactor. *In Vitro Cell Dev Biol Anim*. 2001;37:141.
37. Razian G, Yu Y, Ungrin M. Production of large numbers of size-controlled tumor spheroids using microwell plates. *J Vis Exp*. 2013;81, e50665. https://doi.org/10.3791/50665.
38. Itaka K, Uchida S, Matsui A, et al. Gene transfection toward spheroid cells on micropatterned culture plates for genetically-modified cell transplantation. *J Vis Exp*. 2015;101, e52384. https://doi.org/10.3791/52384.
39. Gonzalez-Fernandez T, Tenorio AJ, Leach JK. Three-dimensional printed stamps for the fabrication of patterned microwells and high-throughput production of homogeneous cell spheroids. *3D Print Addit Manuf*. 2020;7:139–147. https://doi.org/10.1089/3dp.2019.0129.
40. Valdoz JC, Jacobs DJ, Cribbs CG, et al. An improved scalable hydrogel dish for spheroid culture. *Life*. 2021;11:517. https://doi.org/10.3390/life11060517.
41. Fernandes KJL, McKenzie IA, Mill P, et al. A dermal niche for multipotent adult skin-derived precursor cells. *Nat Cell Biol*. 2004 Nov;6(11):1082–1093. https://doi.org/10.1038/ncb1181.
42. Nanduri LS, Lombaert IM, van der Zwaag M, et al. Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. *Radiother Oncol*. 2013;108:458–463. https://doi.org/10.1016/j.radonc.2013.05.020.
43. Anada T, Fukuda J, Sai Y, Suzuki O. An oxygen-permeable spheroid culture system for the prevention of central hypoxia and necrosis of spheroids. *Biomaterials*. 2012 Nov;33(33):8430–8441. https://doi.org/10.1016/j.biomaterials.2012.08.040.
44. Murphy KC, Hung BP, Browne-Bourne S, et al. Measurement of oxygen tension within mesenchymal stem cell spheroids. *J R Soc Interface*. 2017;14, 20160851. https://doi.org/10.1098/rsif.2016.0851.
45. Sarkar S, Peng C-C, Kuo CW, et al. Study of oxygen tension variation within live tumor spheroids using microfluidic devices and multi-photon laser scanning microscopy, 2018 Aug 24 *RSC Adv*. 2018;8:30320–30329. https://doi.org/10.1039/C8RA05505J.
46. Chen K, Li X, Li N, et al. Spontaneously formed spheroids from mouse compact bone-derived cells retain highly potent stem cells with enhanced differentiation capability. eCollection 2019 *Stem Cell Int*. 2019;2019, 8469012. https://doi.org/10.1155/2019/8469012.
47. Li N, Dai X, Yang F, et al. Spontaneous spheroids from alveolar bone-derived mesenchymal stromal cells maintain pluripotency of stem cells by regulating hypoxia-inducible factors. *Biol Res*. 2023;56:17. https://doi.org/10.1186/s40659-023-00421-w.
48. Saito S, Lin Y-C, Tsai M-H, et al. Emerging roles of hypoxia-inducible factors and reactive oxygen species in cancer and pluripotent stem cells. *Kaohsiung J Med Sci*. 2015;31:279–286. https://doi.org/10.1016/j.kjms.2015.03.002.
49. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell*. 2010 Oct 22;40(2):294–309. https://doi.org/10.1016/j.molcel.2010.09.022.
50. Itoh M, Nakayama K, Noguchi R, et al. Scaffold-free tubular tissues created by a bio-3D printer undergo remodeling and endothelialization when implanted in rat aortae. eCollection 2015 *PLoS One*. 2015;10, e0136681. https://doi.org/10.1371/journal.pone.0136681.
51. Bartosh TJ, Ylöstalo JH, Bazhanov N, Kuhlman J, Prockop DJ. Dynamic compaction of human mesenchymal stem/precursor cells into spheres self-activates caspase-dependent IL1 signaling to enhance secretion of modulators of inflammation and immunity (PGE2, TSG6, and STC1). *Stem Cell*. 2013;31:2443–2456. https://doi.org/10.1002/stem.1499.
52. Mittler F, Obeïd P, Rulina AV, Haguet V, Gidrol X, Balakirev MY. High-content monitoring of drug effects in a 3D spheroid model. eCollection 2017 *Front Oncol*. 2017;7:293. https://doi.org/10.3389/fonc.2017.00293.
53. Dong H, Li X, Chen K, Li N, Kagami H. Cryopreserved spontaneous spheroids from compact bone-derived mesenchymal stromal cells for bone tissue engineering. *Tissue Eng C Methods*. 2021;27:253–263. https://doi.org/10.1089/ten.TEC.2021.0001.
54. Zhu J, Zhang S, Jin S, et al. Endochondral repair of jawbone defects using periosteal cell spheroids. *J Dent Res*. 2024;103:31–41. https://doi.org/10.1177/00220345231205273.
55. Mehrotra P, Tseropoulos G, Bronner ME, Andreadis ST. Adult tissue-derived neural crest-like stem cells: sources, regulatory networks, and translational potential. *Stem Cell. Transl. Med.* 2020;9:328–341. https://doi.org/10.1002/sctm.19-0173.
56. Fournier BP, Ferre FC, Couty L, et al. Multipotent progenitor cells in gingival connective tissue. *Tissue Eng*. 2010;16:2891–2899. https://doi.org/10.1089/ten.TEA.2009.0796.
57. Xu X, Chen C, Akiyama K, et al. Gingivae contain neural-crest- and mesoderm-derived mesenchymal stem cells. *J Dent Res*. 2013;92:825–832. https://doi.org/10.1177/0022034513497961.
58. Bardag Gorce F, Al Dahan M, Narwani K, et al. Human oral mucosa as a potentially effective source of neural crest stem cells for clinical practice. *Cells*. 2023;12:2216. https://doi.org/10.3390/cells12182216.
59. Ahmad Zawawi SS, Salleh EA, Musa M. Spheroids and organoids derived from colorectal cancer as tools for in vitro drug screening. *Explor Target Antitumor Ther*. 2024;5:409–431. https://doi.org/10.37349/etat.2024.00226.
60. Takebe T, Sekine K, Enomura M, et al. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature*. 2013;499:481–484. https://doi.org/10.1038/nature12271.
61. Takebe T, Enomura M, Yoshizawa E, et al. Vascularized and complex organ buds from diverse tissues via mesenchymal cell-driven condensation. *Cell Stem Cell*. 2015;16(5):556–565. https://doi.org/10.1016/j.stem.2015.03.004, 7.
62. Wang Y, Sun Y. Engineered organoids in oral and maxillofacial regeneration. eCollection 2023 Jan 20 *iScience*. 2022;26, 105757. https://doi.org/10.1016/j.isci.2022.105757.
63. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*. 2009;459:262–265. https://doi.org/10.1038/nature07935.
64. Takebe T, Sekine K, Kimura M, et al. Massive and reproducible production of liver buds entirely from human pluripotent stem cells. *Cell Rep*. 2017;21:2661–2670. https://doi.org/10.1016/j.celrep.2017.11.005.
65. Bouchi R, Foo KS, Hua H, et al. FOXO1 inhibition yields functional insulin-producing cells in human gut organoid cultures. *Nat Commun*. 2014;5:4242. https://doi.org/10.1038/ncomms5242.
66. Takasato M, Er PX, Chiu HS, et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature*. 2015;526:564–568. https://doi.org/10.1038/nature15695.
67. Gramatiuk SM, Ivanova YV, Hudyma AA, Sargsyan K, Kryvoruchko IA, Puliaieva IS. Differentiation of neurosphere after transplantation into the damaged spinal cord. *J Med Life*. 2023;16:689–698. https://doi.org/10.25122/jml-2022-0346.
68. Assinck P, Sparling JS, Dworski S, et al. Transplantation of skin precursor-derived schwann cells yields better locomotor outcomes and reduces bladder pathology in rats with chronic spinal cord injury. *Stem Cell Rep*. 2020;15:140–155. https://doi.org/10.1016/j.stemcr.2020.05.017.
69. Ohazama A, Sharpe PT. TNF signalling in tooth development. *Curr Opin Genet Dev*. 2004;14:513–519. https://doi.org/10.1016/j.gde.2004.07.008.
70. Ohazama A, Modino SA, Miletich I, Sharpe PT. Stem-cell-based tissue engineering of murine teeth. *J Dent Res*. 2004;83:518–522. https://doi.org/10.1177/154405910408300702.
71. Modino SA, Sharpe PT. Tissue engineering of teeth using adult stem cells. *Arch Oral Biol*. 2005;50:255–258. https://doi.org/10.1016/j.archoralbio.2005.01.002.
72. Nakao K, Morita R, Saji Y, et al. The development of a bioengineered organ germ method. *Nat Methods*. 2007;4:227–230. https://doi.org/10.1038/nmeth1012.
73. Tanaka J, Ogawa M, Hojo H, et al. Generation of orthotopically functional salivary gland from embryonic stem cells. *Nat Commun*. 2018;9:4216. https://doi.org/10.1038/s41467-018-06469-7. PMID: 30310071; PMCID: PMC6181987.
74. Tanaka J, Senpuku H, Ogawa M, et al. Human induced pluripotent stem cell-derived salivary gland organoids model SARS-CoV-2 infection and replication. *Nat Cell Biol*. 2022;24:1595–1605. https://doi.org/10.1038/s41556-022-01007-6.
75. Kim EJ, Yoon KS, Arakaki M, et al. Effective differentiation of induced pluripotent stem cells into dental cells. *Dev Dynam*. 2019;248:129–139. https://doi.org/10.1002/dvdy.24663.