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Production of Cartilaginous Organoids: Potential Opportunities and Challenges, A Review Article

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Abstract

Damaged articular cartilage has limited self-healing potential and often leads to osteoarthritis (OA), pain, and dysfunction of the affected joint. Autologous and allogenic transplants cannot fully meet the needs of clinical treatment. Two dimensional (2D) and three-dimensional (3D) cell cultures can help to study growth modeling and physiological characteristics of the human body. Among the problems that 2D and single-layer cultures have the lack of proper and accurate tissue modeling and the lack of tissue complications similar is to the original tissue. With organoid models, cellular and tissue structural studies and functional and physiological studies of tissues have been revolutionized and more accurate. Organoids are useful for studying repair and drug efficacy. Physiological and pathological investigations by combining *in vitro* and *in vivo* methods have become more effective today. The purpose of this study is to investigate the factors involved in the formation of cartilage organoids so that we can introduce the best method of organoid production for the healing of cartilage damage by using cell types and organoid model.

Keywords: Cartilage, 3D culture, Organoids, Osteoarthritis

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Introduction

The elastic and flexible tissue of cartilage is one of the vital tissues that exist in the internal and external organs of animals and humans (1). Cartilage assists in the mechanical movements of the body and in addition, allows the bones to move without touching each other (2). In general, the cartilage tissue is divided into three main categories, including hyaline, elastic, and fibrous, which are found throughout the body such as the joints and growth plates, nose, ribs, and airways (3). All these types have low cell densities of chondrocytes which are speeded in the extracellular matrix (ECM) (4). The articular cartilage lacks blood vessels and nerves, so natural cartilage regeneration is limited. The structure of the ECM of the cartilage contains abundant proteoglycans, which are placed in a network of hydrated fibrillar collagen (5). The amount, distribution, collagen fibers, and ECM compounds are different in tissues; this causes differences in their appearance and biomechanical properties (6). The survival of chondrocytes is essential to maintain the integrity of articular cartilage (7). Chondrocytes can maintain a dynamic balance between ECM production and enzymatic degradation. Loss of catabolic balance leads to the destruction of articular cartilage seen in osteoarthritis (OA) (6). The most important and common joint disease and injury in the elderly is OA (8), which causes significant pain and disability. The reason for OA is intricate and various, containing a range of biomechanical, biochemical, and genetic factors (9). Destruction and loss of articular cartilage in OA leads to bone spurs and cysts at the periphery of the joint (10).

One of the important tools used to study cell and tissue activities is the use of various cell culture methods; this method provides the possibility of studying and comparing protein extraction and production, tissue shape, and cell activity. Inconsistency in signaling close dissimilarity in cell interactions and different cell morphology and change in cell polarity can be introduced as the most important differences between the two-dimensional (2D) and 3D cultures. Due to the mentioned problems in 2D cultures, conditions were provided for the development of cultures that have a closer resemblance to the body environment3 .D cultures can create a more accurate and appropriate understanding of the pathology of various diseases by optimizing the conditions of cell culture and also being effective in more efficient and targeted treatment (11). There are several methods to create a 3D culture that can be based on scaffolds or without the use of scaffolds. Scaffolds can be derived from natural or synthetic materials (12).

Organoids refer to cells that grow in a 3D environment

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to form small assemblies, which have the ability to self-organize and have the power to differentiate and create functional structures (13). These structures are also called small organs because they represent the function of body organs which have great potential for modeling tissue growth and disease, and for personalized medicine, drug screening, and cell therapy (13-15). The 3D structure of the organoid supports the stem and precursor cells that have the ability to reproduce the functions of organs and also provide a structure similar to the original tissue (16). Pluripotent induced cells (PSCs) and a variety of embryonic and adult stem cells (ASCs) can produce organoids. Organoids formed from these cells are used to simulate and study the heart, intestine, liver, lung, brain tissues, etc. (17-19). 3D cultures are older than organoids, and the most important advantage of organoids is their coordination and combination with tissue engineering to imitate the desired tissue (20); they include cells with different characteristics that can be used for modeling disease and tissue growth (21, 22). In studies of organoids, and tissue microenvironments, various factors are effective in the homeostasis of the body, one of the most important of which is the communication between cells and the ECM. Bioengineering techniques lead to direct cellular communication and behavior by analyzing how organs function and reconstructing the systems. It can be mentioned that 3D culture techniques are a way to create organoids (23). The purpose of this study is to investigate the factors involved in the formation of cartilage organoids so that we can introduce the best method of organoid production for the healing of cartilage damage by using cell types and organoid model.

Literature search method

In this review, the following words and phrases were used to search and obtain articles from different databases: organoids and stem cells, cartilage and cartilage diseases, and cartilage signaling. Also, PubMed and Science Direct sites and Scopus search engines were used for the systematic review of PRISMA protocols. Duplicate articles were removed, and studies focused on their titles were first reviewed; then, their abstracts were screened. Also, if the connection between organoids and cartilage systems was not related to repair, they were excluded from the study. All studies were selected in English and no other languages were included. In addition, the information that was considered for each article was the language of the article, the time of publication, the type of model, and whether it is human or animal.

Cartilage development and microenvironment

The skeletal structure of the body is mainly composed of cartilage and bone. During the embryonic stage and the time of production of cartilage progenitor cells, three cell lineages have the ability to differentiate into cartilage cells, which are paraxial mesoderm sclerotome and notochord (24). It increases the expression of adhesion molecules such as cadherin or immunoglobulins. Various factors affect the function of cartilage, which is regulated by cytokines and different signaling pathways, which are effective in the proliferation and differentiation of cells towards the tissue and cartilage cells. Different cytokines such as transforming growth factor beta (TGFβ) and Wnts and fibroblast growth factors (FGFs) are involved in cartilage growth (25-27). These cytokines are essential molecules in cell-cell interactions that affect *Sox9* gene expression and are critical for cartilage development (Fig.1) (24, 28).

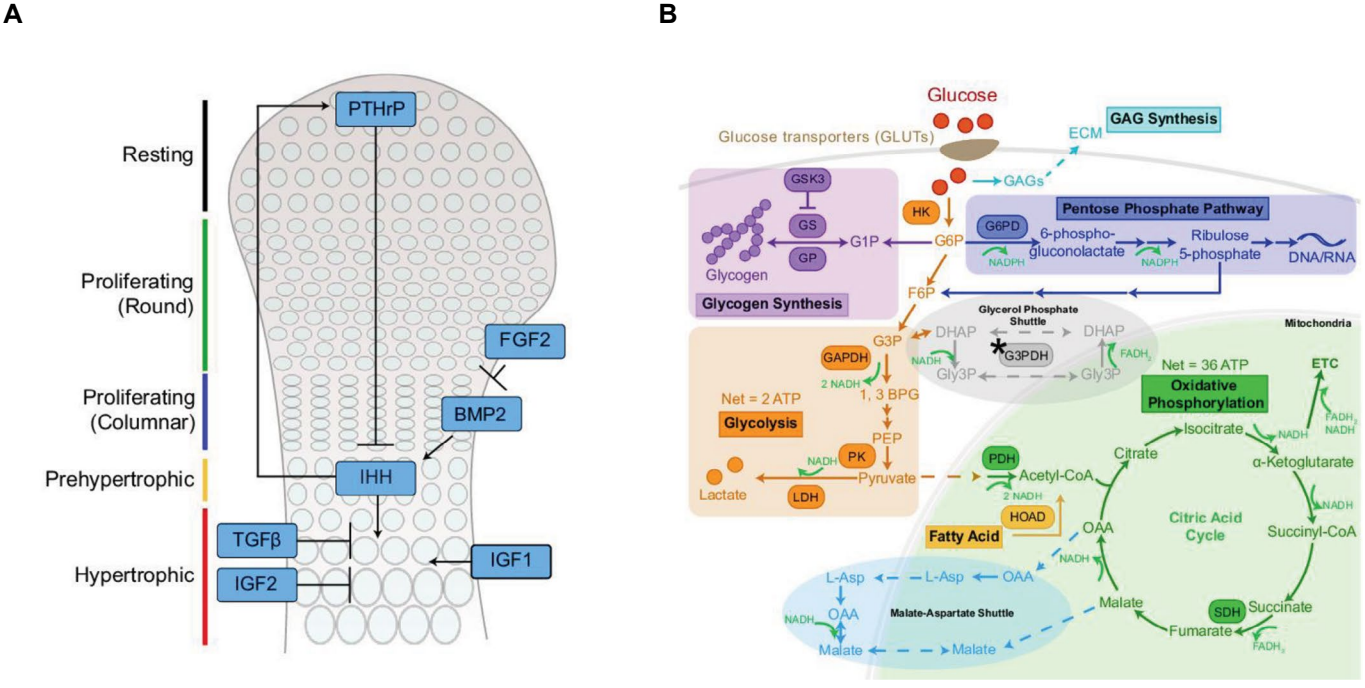

Fig.1: Cellular mechanism involved in cartilage development and microenvironment (24, 28). **A.** Chondrocyte development in growth plates is regulated by signals like PTHrP, IHH, WNTs, FGFs, BMPs, TGFβ, and IGFs and **B.** Glucose metabolism in cells involves multiple pathways, with G6P as a central molecule. Electron transfer to mitochondria for ATP production occurs via the malate-aspartate shuttle, with the glycerol phosphate shuttle being absent in chondrocytes.

Signaling molecules involved in chondrogenesis

Various factors influence the density and uniformity of cartilage tissue, and cell-to-cell communication is one of the factors involved. Among the important adhesive factors in this factor are TGFs, FGFs, SMADs, and MAPKs which are important molecules in cell adhesion. Other factors in the growth and activity of the cartilage in developing structures include Hox and LEF-1 transcription factors necessary for organogenesis (28).

The interaction of Wnts and Hox transcription factors of organ mesenchymal cells and factors secreted from the organ epithelium can affect the growth of organs. TGF transcription factor is one of the regulatory factors of Hox and Shh responses to form the ECM (28).

Wnts in chondrogenesis

The Wnt family is involved in organogenesis and organ development. For intrachondral ossification, the Wnt signaling pathway is essential, and this pathway depends on β-catenin. Excessive activity of the WNT signaling pathway can cause incomplete growth and prevent the formation of primary cartilage; it is also effective in disrupting the organization of the growth plate. Genetic studies show the destructive and inappropriate effect of Wnt/β-catenin overexpression, which can be harmful for the treatment of OA. Also, in addition to cartilage, overexpression of Wnt/β-catenin can have a destructive effect on the synovium and bone (Fig.2) (29).

For the first time, growth and maturation of the cartilage and its relationship with the WNT signaling pathway were identified in Wnt-Frzb/Frp3/Sfrp3 studies in chickens (30, 31). Wnt has a diverse number of families that have different and specific functions; for example, Wnt4 and Wnt14 are found in the cartilage and joints. Or, for example, Wnt5a is found in the epiphyseal region of the bones in the cartilage growth plates, and Wnt5b is found in pre-hypertrophic cartilages. During embryonic development, the presence of Wnt11 has been confirmed by examining the perichondrium (32).

Overexpression of Wnt5a, 5b, or Frzb/Frp3/Sfrp3 in growing or cultured organ buds indicates cartilage stimulation, and differentiation of the mesenchymal cells into cartilage cells, whereas overexpression of Wnt1, Wnt4, or Wnt 8A inhibits the cartilage formation, In addition to the actions of single Wnts on the cartilage formation, studies have shown that overexpression of the mutant activator of β-catenin stimulates cartilage cell hypertrophy and endochondral ossification in the growing organs of chickens (24). In contrast, overexpression of Frzb and Wnt5a inhibits cartilage hypertrophy, matrix mineralization, and endochondral ossification (33).

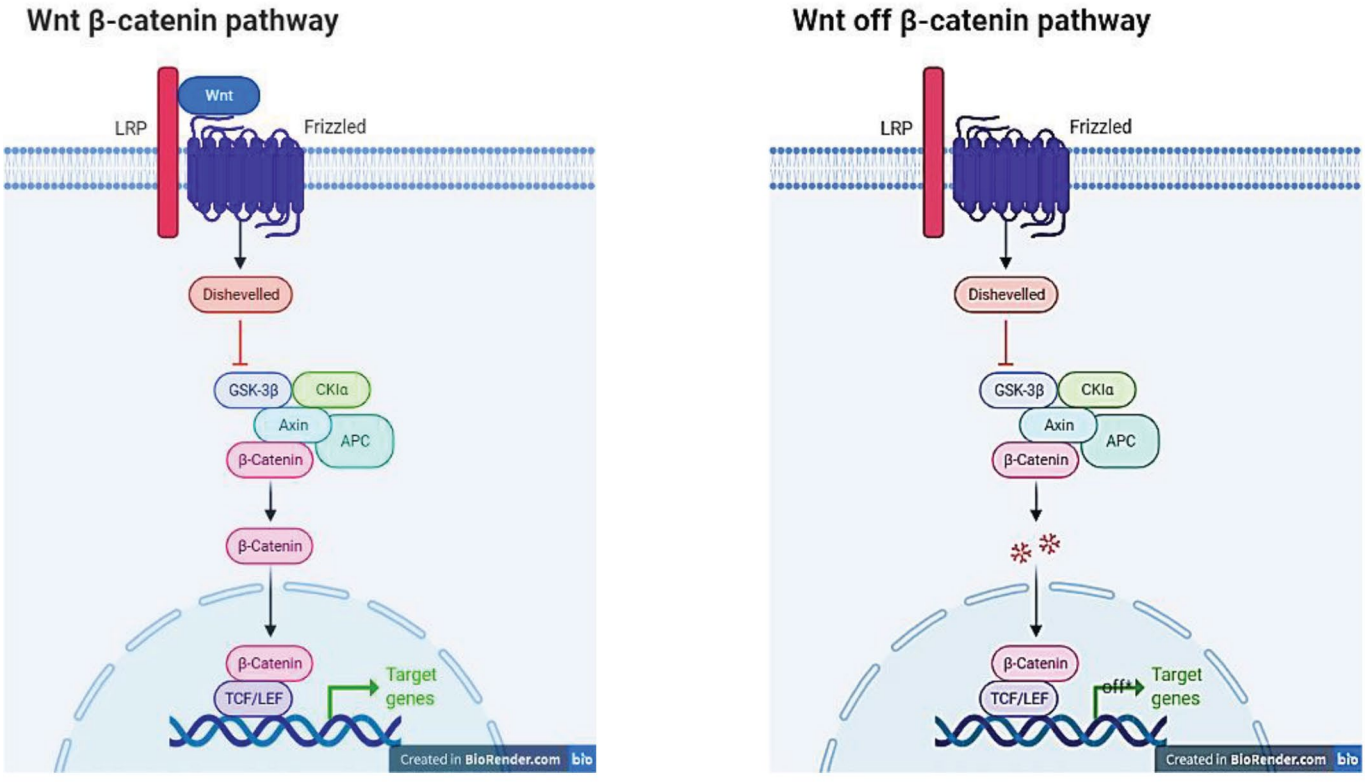

Fig.2: WNT/β-catenin signaling pathway. Unconventional pathway (right): β-catenin phosphorylation by glycogen synthase kinase 3β (GSK-3β), auxin, adenomatous polyposis coli (APC) and casein kinase-1 (CK-1) complex. Active pathway: (left): WNT binding to its receptor, followed by the activation of DSH protein (LRP5/6) by forming a complex, helps to phosphorylate β-catenin (29).

Important pathways are used in the regulation of the growth and formation of cartilage, bone, and joints, but the Wnt/β-catenin signaling pathway plays a vital role in the homeostasis and biological function of these structures, which helps to maintain the integrity of the cartilage ECM structure and the differentiation of stem cells. It is also necessary to establish phenotype coordination and maturation of hypertrophic tissue during osteogenesis in the cartilage (34).

TGFβs signaling in cartilage development

One of the molecules that regulates different cell pathways is the large family of TGFβ, which plays a role in the control and regulation of cartilage cells, density, proliferation, and maintenance of the articular cartilage cells (24). Three types of TGFβ have been identified in the chondrocytes and mesenchymal cells, which are types 1, 2, and 3 in joints and type 3 in elastic cartilages such as ribs. With the studies conducted to identify and influence TGFβ on cartilages, it has been observed that in the perichondrium, TGFβ3 is expressed at a much higher rate than other TGFβ groups, while in the epiphyseal regions, which are related to growth, TGFβ1 and TGFβ3 are expressed in areas of cartilage that have been proliferative and hypertrophic (35, 36). The TGFβ family has a large collection that includes activin (A and B), nodules, myostatin (GDF-8), and Mularine BMP inhibitors.

Ligands are classified based on their downstream signaling mediators, which are Smads (25, 37). The main mechanism of signal transmission in the TGFβ family is by R-Smads type 2 and 3, and the mediator of BMP signal transmission can be introduced by R-Smads type 1, 5, and 8. R-Smads are essential for signaling. TGFβ function is regulated by R-Smads which are involved in proliferation, differentiation, apoptosis, cell migration, and ECM destruction (38).

When TGFβ ligands bind to their main receptors on the cell surface, they activate signaling cascades inside the cell (Fig.3). The most important cell surface receptors of TGFβs are serine/threonine kinase type I and II (24). When the ligand binds to the receptor on the cell surface, it activates serine/threonine kinase type II, which trans-phosphorylates the type 1 receptor (39). The main signaling pathway in TGFβ is through Smad, in which R-Smads 1.5 and 8 connect to BMP and ALK1, 2, and 3 receptors and send signals (40). With the presence of MISRII, the phosphorylation of R-Smads by type I receptors begins, where signals are transmitted to the nucleus to cause transcription (Fig.3).

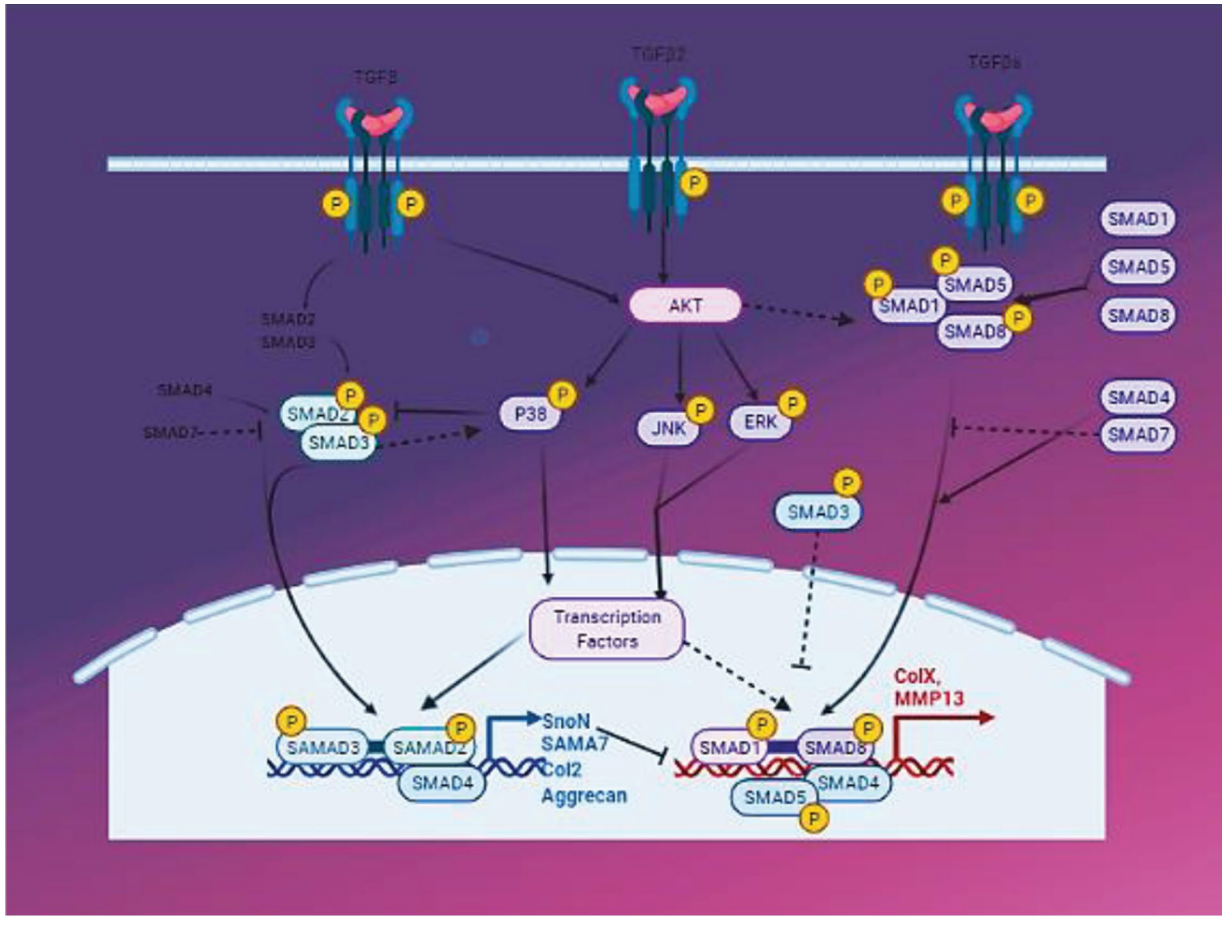

Fig.3: Conventional and unconventional transforming growth factor beta (TGFβ) signaling pathways in the cartilage.

Sources of cartilage cells and the generation of cartilage organoids

Stem cells are the main source for making organoids. Among the most important challenges of using organoids, we can mention the difficult control of cell-cell/cell-matrix communication in the structure, which indicates that it is possible to control the tissue (20). Organoid systems are located in environments which are effective in improving the activity and functions of stem cells (41).

In addition to stem cells, chondrocytes can be used to produce cartilage organoids. One of the first effective methods in the reconstruction and improvement of articular cartilage is the use of autologous chondrocyte cells. They are used to collect and obtain articular cartilage samples from areas that bear less weight, such as patellas, and they are transferred to laboratories in order to increase their number (42). Binding to the integrin receptor by various components of the ECM, including collagens and fibronectins, provides conditions for changes in cell behavior (43).

To differentiate the cartilage from mesenchymal stromal cells (MSCs) *in vitro*, undifferentiated MSCs express type I collagen, hyaluronan, tenascin-C, and fibronectin. In this way, with the cooperation of molecular mediators that play a role in regulating morphogenetic pathways, for example, heparan sulfate, cadherin, and chondroitin sulfate, cells begin to become cartilage precursors. By expressing the genes *SOX trio, L-SOX5, SOX6*, and *SOX9*, it is possible to produce different components of the cartilage ECM, such as collagen II and IX proteins; with increasing the ECM production, chondroblast cells are enclosed in self-secreted matrix and the phenotype and obtain the morphology of cartilage cells. But in the center, there is little differentiation and the cells are enclosed in a matrix containing collagen type II, aggrecan, and glycosaminoglycan (GAG) (Table 1) (44).

Various cells can be used as a source to create organoids, the most widely used of which are PSCs and ASCs, which mimic the biochemical and physical conditions of natural tissue and cause tissue production growth and cellular homeostasis. When they have a suitable 3D scaffold, the conditions for the differentiation of stem cells are provided, and they can create different tissue organoids (45).

The applications of multifunctional cartilage organoids

Drug screening

According to different methods in cell culture, due to the suspicion of organoids with the main structure that exists in the body, these organoids have become the main technique for most cell studies (46). Various types of body tissues, and 3D physiological architecture, with the help of organoid systems, create powerful technologies for biological and clinical applications (Fig.4) (13).

The disease modeling of the cartilage tissue structures by cartilage organoids can be used as useful modeling tools and powerful research platforms for drug screening by identifying the key signaling pathways during embryogenesis and cartilage regeneration. Cartilaginous cells in an organoid are of fundamental clinical importance in the repair of various cartilage structures throughout the body. They are used to investigate drug toxicity and personal medicine and studies related to the response of the patient receiving cell therapy with the drug used and the effect of the disease on the host (47). The use of microfluidics and organ-on-a-chip along with cartilage organoids can provide high-performance drug screening and create suitable conditions for the production of these organoids (48).

Disease model

Cartilage organoid models include multicellular components and cartilage extracellular matrices, and effective cartilage organoids are used in personalized medicine and new treatment and diagnosis strategies (47). In cartilage regeneration, significant cartilage-like tissue regeneration with a limited number of cells is important. The indoor organoid method prevents growing spontaneously with ECM accumulation. Therefore, it will be necessary to convert the closed space cultivation technique to the open space cultivation technique (49).

In the studies that have used cartilage organoids, the evidence of the growth and maturity of the created cartilage is shown. By using these organoids, they produce "mini-joints" that are used in the investigation and study of cartilage and inflammatory diseases, as well as the investigation of agonists. Adenosine receptors have been used. The results indicate that these organoids are effective in tissue and disease studies (50).

Overview and future directions

Progenitor cells and stem cells can have great potential in cell transplant applications and are a suitable alternative for cartilage regeneration. Due to the fact that there are many sources, the sources are PSCs or iPSCs, but PSCs are the cells that are difficult to extract and obtain; for this reason, using iPSCs is a more logical solution for creating organoids because both can be used as autologous and applied for more people. To create a more accurate model, 3D bioprinting is an effective method in creating a more accurate size and number of cells; a structure similar to the cartilage is effective and various factors are involved in creating a 3D model, each of which has several challenges (51).

One of the hopes of cell therapy and regenerative medicine is the use of organoids in different tissues, which can be used to regenerate the cartilage, and 3D cultures and organoids can be promising for the treatment of injuries, which can open a new path for the diagnosis and treatment of cartilage injuries. Among the applications that we can introduce for cartilage organoids is the investigation of the physiological functions of the cartilage and their signaling pathways, and exploratory investigations on rare diseases, for example, related to cartilage genes, as well as personal medicine. Screening of drugs with high effectiveness and efficiency is recommended (52).

Table 1: Examples of methods and research that have been studied for organoid cartilage

Types of stem cells	Species	Organoid identity	Starting cells	Intrinsic patterning or extrinsic signaling molecules	Extracellular scaffold and/ or bioreactor	High-throughput analysis	Special considerations for cartilage modeling	Reference
SMSC	Human	SMSC organoids	SMSC	*FOXC1, HIF1α, SOX9*	3D-cultured	miRNA and mRNA sequencing	Serving as a model to study adult cartilage homeostasis and disease *in vitro*	(53)
Chondrocytes	Primary rat-human	Organoid cartilage	Costal chondrocytes	Collagen	Fiber/ chondrocyte-organoid culture	Gene expression mRNA sequencing	Development of an articular cartilage graft	(49)
Chondrocytes	Bovine	Organoid cartilage	Chondrocytes	Called pericellular matrix (PCM)-*KI67*	Hydrogel alginate	mRNA sequencing	It includes the increase in the volume of cartilage cells, organoid formation and their accumulation in neo hyaline cartilage.	(54)
Pluripotent stem cells (iPSCs)	Human	Organoid cartilage	hESCs	*SMAD, BMP, TGF-β, FGF8*	Hydrogels	Spectrometry cartilage proteins mRNA sequencing	The main reason for the congenital malformations of the skull and face and the reconstruction of the cartilaginous structures of the head and face	(55)
iPSCs	Rat	Organoid bone	Bone stem cell	*RUNX2, OSX, Sox9*	NA	DNA quantification	Considered as a living “bio-essence” that allows the production of tissues with specific geometrical features and specific internal features	(56)
Chondrocytes	Bovine	Articular Cartilage	Articular cartilage		NA	mRNA gene expression	The spherical organoid is suitable for understanding the mechanism of histogenesis and pathogenesis in articular cartilage.	(57)
Chondrocytes	Mouse, human, and pig	Organoid cartilage	Bone stem cell articular cartilage	*Sox9*	*In vitro* culture	RNA sequencing	It can be used to investigate the effects of surrounding signals on cartilage cells	(58)
iPSCs	Human	Callus organoids	hPDCs	*Sox9*	*In vitro* culture	RNA sequencing	Regeneration of bone and cartilage	(59)
iPSC	Rat	Cartilage organoids	Periosteum-derived cells	*SOX9*	Hydrogels	mRNA gene expression	Use of pre-programmed living building blocks.	(60)

SMSC; Synovial mesenchymal stromal cell, iPSCs; Pluripotent stem cells, hESCs; Human embryonic stem cells, hPDCs; Human periosteum-derived cell, NA; No scaffolding, and OA; Osteoarthritis.

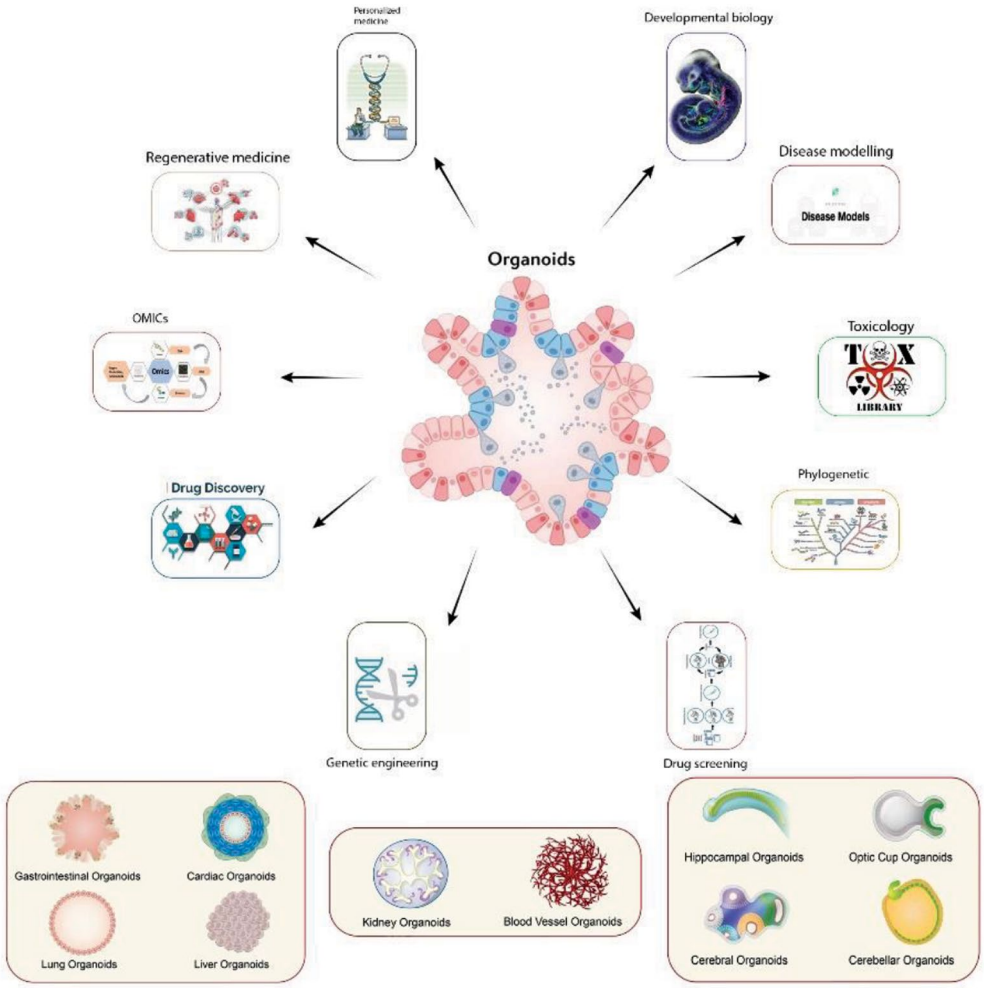

Fig.4: Organoids hold significant potential for various applications, including basic research, drug screening, genomic and metabolomic analysis, and gene editing to investigate disease-associated alleles through genetic engineering. Additionally, organoids can serve as disease models, play a role in cell immunotherapy and regenerative medicine, and offer the possibility of establishing biobanks.

With all the advances that have been made in the field of stem cells and working with organoids, a full and correct understanding of their growth and functionality in living organisms is still not complete (51). Autologous cartilage implantation using extracellular cartilage cells was the first cartilage cell therapy. One of the limitations of these cells was the low potential of the chondrocytes, which led to the study of mature MSC as a replacement cell source. Another source that can be used as a suitable cell source for the creation of cartilage organoids is embryonic cartilage cells that can produce chondrocytes with a phenotype close to articular cartilage, but the preparation of these cells is difficult and has ethical problems (61).

One of the challenges of advancing cartilage organoid systems is the optimization of their culture system because it is necessary to transfer it from the laboratory to clinical and human use. Another challenge of working with organoids is a function similar to that of tissues, and the length of time an organoid can remain in the body and be functional should be investigated (51). Development of better proxies for tissues and organs *in vivo* and compatibility of multi-tissue organoids in several larger systems (such as humans on a chip) are needed (20).

Challenges of organoid applications

One of the most important uses of organoids is the possibility of personalizing treatments. In general, the biological characteristics of donor cells are the same for organoids, but there are differences in non-genetic characteristics such as living environment and lifestyle. They are of different ages (62).

Another problem that can arise in cultivation is that in 2D cultures, the phototype of the cells differs, which can affect the organoid. Another challenge that arises in organoid systems is the clonal diversity of primary cells. Then, there are cells derived from these cells that are effective in the differentiation of organoids (52).

Another challenge is the lack of clear evidence on the effect of epigenetics on iPSCs and organoids formed from these cells, and the length of time the effect of this epigenetic can remain stable (63).

Another challenge of organoid systems is the need for

universal agreement and the existence of global standards in this field, completely adjusted based on the application of each organoid (64).

Conclusion

The production of cartilaginous organoids represents a significant advancement in the field of tissue engineering and regenerative medicine. These three-dimensional structures, which mimic the complex architecture and function of native cartilage, offer promising opportunities for studying cartilage development, disease modeling, and drug testing. Moreover, they hold potential for clinical applications in repairing cartilage damage and treating degenerative conditions such as OA.

One of the primary opportunities provided by cartilaginous organoids is their ability to serve as accurate *in vitro* models. These models can improve our understanding of cartilage biology and pathophysiology, enabling the discovery of new therapeutic targets. Additionally, the potential for patient-specific organoids paves the way for personalized medicine approaches, where treatments can be tailored to the individual's unique cellular makeup.

Despite these promising opportunities, several challenges must be addressed to fully realize the potential of cartilaginous organoids. Key among these challenges is the need for standardized and reproducible methods of organoid production. Variability in cell sources, differentiation protocols, and culture conditions can lead to inconsistencies in organoid quality and function. Moreover, achieving the mechanical properties and long-term stability of native cartilage remains a significant hurdle.

Another challenge lies in the integration of vascular and neural components into cartilaginous organoids. Native cartilage is avascular and aneural, but for larger and more complex tissue constructs, incorporating these elements may be necessary to ensure proper nutrient delivery and integration with host tissues post-implantation.

Furthermore, scaling up the production of cartilaginous organoids for clinical use presents logistical and regulatory challenges. Ensuring the safety, efficacy, and ethical considerations of organoid-based therapies will require rigorous testing and adherence to regulatory standards.

In conclusion, while the production of cartilaginous organoids holds immense potential for advancing both research and clinical applications, overcoming the associated technical, biological, and regulatory challenges is crucial. Continued interdisciplinary efforts in bioengineering, materials science, and clinical research will be essential to harness the full benefits of this promising technology.

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Authors' Contributions

A.R., H.D.; Conceptualization and Methodology. M.P.; Data curation and Writing-original draft preparation. A.R.; Supervision. F.Z., A.R., H.D.; Writing. S.D.; Reviewing. S.I., M.D.; Editing. All authors read and approved the final manuscript.

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