Histol Histopathol (2021) 36: 1219-1234

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

REVIEW **Open Access**

Is extracellular matrix (ECM) a promising scaffold biomaterial for bone repair?

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Summary. The increasing demand for bone grafts and the scarcity of donors worldwide are promoting researchers to seek alternatives. The extracellular matrix (ECM) has been reported to enhance properties of osteoconduction and osteoinduction by simulating the molecular structure of bone and facilitating cell infiltration for bone repair. As one of several novel biomaterials, ECM has many desirable properties, including biocompatibility, bioactivity, and biosafety. Thus, we evaluated whether ECM is a promising scaffold biomaterial for bone repair. In this review, we explore ECM composition, the sources and fabrication methods, especially the decellularization technique, of ECM scaffolds. Furthermore, we highlight recent progress in the use of ECM as a scaffold biomaterial for bone repair. Generally, ECM is used in 1) three-dimensional (3D) cell cultures to promote osteogenic differentiation, 2) combinations with other biomaterials to increase their osteogenic effects, 3) 3D printing to produce customized or patient-tailored scaffolds for bone repair, and 4) hydrogels derived from ECM used for bone repair. In addition, we focus on future prospects for application of ECM as a scaffold material used for bone repair. From this review, we expect to have a perfect understanding of ECM-based scaffold materials in the hope that this leads to further research of the production of ECM biomaterials to meet the clinical needs for bone repair.

Key words: Extracellular matrix (ECM), Scaffold biomaterials, 3D cell culture, 3D printing, Bone repair

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DOI: 10.14670/HH-18-370

Introduction

Massive bone defects caused by tumors, trauma, infections, or congenital disorders present a major clinical problem. It was estimated that at least two million bone grafting surgeries worldwide were performed to cure major bone defects (Sohn and Oh 2019; Busch et al., 2020). Bone grafts are the second most common type of transplantation, following blood transfusion (Sarkar et al., 2019). Currently, autografts and allografts are the two main therapeutic methods for treating bone defects (Bai et al., 2018). Autologous bone grafting has long been considered the gold standard for bone reconstruction because of its excellent osteoconductivity, osteoinduction, and immune-compatibility (Wang and Yeung, 2017; Lobb et al., 2019). However, the limited availability of bone material and the associated complications, including pain, blood loss, infection, and fracture at the bone extraction sites restrict the clinical applications of autologous bone grafts (Busch et al., 2020; Cha et al., 2020; Hofmann et al., 2020). Alternatively, allogenic bone grafts, which have similar mechanical properties and biological activity to autologous bone, have become widely used in bone reconstructive surgeries (Salamanna et al., 2020). Nevertheless, in addition to the limited sources of allografts, there are potential risks of a host immune response, infection, and pathogenic virus transmission (Cheung et al., 2018).

Developments in the technology of bone tissue engineering and orthopedics have been ongoing. Artificial bone can now be customized and mass-produced. Bone tissue engineering has enabled the development of a bone surrogate, which has the ability to repair bone defects following tissue engineering principles and methods (Eivazzadeh-Keihan et al., 2020; Ramirez-Rave et al., 2020). The ultimate goal of bone tissue engineering is to regenerate damaged or defective

bone tissue using osteoconductive and/or osteoinductive scaffolds (Liu et al., 2020; Wang et al., 2020). One of the major developments within the past 20 years has been construction of the basic pattern of tissue-engineered bone, consisting of active osteogenic cells, molecules related to bone regeneration, and scaffold materials for bone repair.

The ideal scaffold material should have a composition similar to that of natural bone, provide similar mechanical strength, and provide a similar biomicroenvironment (Salg et al., 2019). Moreover, it should enable cell recruitment, growth, proliferation, migration, and differentiation, in a manner similar to that of the native ECM. Currently, scaffold materials used in bone tissue engineering can be roughly divided into three categories: 1) natural biological materials such as collagen (He et al., 2019; Ebrahimi et al., 2020), chitosan (Dinescu et al., 2019), and fibrous protein (Li et al., 2019); 2) synthetic inorganic materials such as hydroxyapatite (HAP) (Zhang et al., 2018) and tertiary calcium phosphate (Arpornmaeklong and Pressler, 2018); and 3) synthetic organic polymer materials such as poly-lactide-co-glycolic acid (PLGA) (Thi et al., 2017; Akar et al., 2019), polyhexolactone (PCL) (Venugopal et al., 2019; Yao et al., 2020), and polylactic acid (Gremare et al., 2018). Nevertheless, the majority of these materials do not exhibit osteoinductive capacities but merely have the ability to conduct bone formation (Valtanen et al., 2020). The addition of ceramic particles, growth factors, and ECM peptides or proteins has been used to increase the bioactivity of scaffold materials (So et al., 2020). However, the quantities and optimal combinations of these elements have not been ascertained. These considerations are necessary to generate native ECM-based materials to repair bone defects.

The ECM is an extracellular product secreted by cells in living tissues and organs. It consists of a complicated network of abundant proteins, proteoglycans, and other macromolecules. The ECM can furnish structural and biochemical support to the surrounding cells (Tamburrini et al., 2020). As a microenvironment for cell growth, the ECM plays an important part in cell adhesion, proliferation, migration, and differentiation (Bekku and Oohashi, 2019). The three-dimensional microstructure of the ECM can provide a microenvironment that is very similar to the native growth environment of the cells. This variety of active molecules provides the basis for various cell activities, making the ECM an ideal tissue engineering material (Tamimi et al., 2020). Scaffolds based on natural ECM have been used for regenerating heart valves (Curley et al., 2019; Goldfracht et al., 2019) and trachea (Macchiarini et al., 2008). The ECM has also emerged as a potential off-the-shelf product to produce bone due to its capacity to activate osteoprogenitors and immunoregulation (Garcia-Garcia and Martin, 2019). In addition, some decellularized ECM scaffolds have been commercialized and ratified by the Food and Drug Administration (FDA) for human clinical use. Examples include dermis or skin tissue (Alloderm®; LifeCell), porcine heart valves (Synergraft®; Cryolife), and porcine urinary bladder (ACell) (Cheng et al., 2014; Lee et al., 2018). To our knowledge, there are no reports describing the use of decellularized ECM for bone-specific clinical applications, which may be due to regulatory difficulties, high costs, and speculative advantages.

In this review, we discuss recent advances in ECM composition and fabrication methods, particularly decellularization. We then focus on the application of ECM scaffolds in bone regeneration. We conclude with the current challenges and perspectives of ECM-based scaffolds for bone repair. Our aim with this review was to facilitate the understanding of ECM-based scaffolds to encourage the development and production of ECM biomaterials to meet the clinical needs for bone repair.

Overview of published articles pertaining to ECM scaffolds in bone tissue engineering and bone regeneration

Publications up to 30 March 2020 were retrieved from the PubMed, MEDLINE, and Web of Science databases. ((((ECM) OR (extracellular matrix) OR (decellularized matrix) OR (cell-free matrix)) AND ((scaffold material) OR (scaffold))) OR (decellularized bone matrix)) AND ((bone tissue engineering) OR (bone regeneration) OR (bone defects repairing) OR (bone defects repair) OR (bone defects restoration)). The titles and abstracts of 297 articles were reviewed and selected. In addition, potential missing articles were searched for by reference tracking of the included articles. Ultimately, 224 articles were selected. The retrieval flow chart is shown in Fig. 1. In this review, we first introduced the sources and fabrication methods, especially decellularization, of ECM scaffolds. Next, we focused specifically on ECM scaffolds and their applications in bone repair. The articles we retrieved could be divided into four main categories: 97 articles pertain to the application of ECM scaffolds in three-dimensional (3D) cell culture to promote differentiation of osteogenic cells, 73 discuss the combination of ECM with other biomaterials to increase the osteogenic effect of biomaterials, 49 address the use of ECM in 3D printing, and 51 discuss hydrogels derived from ECM. Finally, we discussed the advantages of using ECM scaffolds for repairing bone defects, in addition to future perspectives and the current challenges of ECM scaffold research.

ECM composition

As described in Fig. 2, the main components of ECM are composed of three macromolecules: proteoglycans, fibrous proteins and viscous proteoglycans (Jarvelainen et al., 2009; Schaefer and Schaefer, 2010). Proteoglycans and fibrous proteins provide the ECM with varied architectures and viscoelasticity. Viscous proteoglycans fill the interstitial

space, serving different functions owing to their unique properties (Padhi and Nain, 2020).

ECM scaffolds

Sources of ECM scaffolds

The components of ECM scaffolds are conserved between homogeneous and heterogeneous individuals; hence, ECM scaffolds hardly display any tissue specificity (Startseva et al., 2019). Moreover, due to the removal of cell surface antigens and all intracellular components by acellular technology, ECM scaffolds have reduced immunogenicity and can be used for both homo- and xenotransplantation. According to our comprehensive evaluation, the source and application fields of ECM scaffolds are very wide. Generally, the tissue sources of ECM scaffolds include human, porcine, bovine, and equine tissues (Keane and Badylak, 2015). Many varieties of decellularized scaffolds have been commercialized and approved by the FDA for clinical use (Mosala Nezhad et al., 2016; Startseva et al., 2019); the details are shown in Table 1.

ECM scaffolds derived from xenografts

ECM scaffolds are typically derived from xenogenous tissues, such as porcine urinary bladder (Huleihel et al., 2017; Ghuman et al., 2018), porcine or bovine small intestine (Sun et al., 2018a,b; Zhang et al., 2019; Xie et al., 2020), cow tendon (Toprakhisar et al., 2018), and porcine skin (Bhattacharjee et al., 2019), after the successful removal of antigens by acellular techniques. For example, a group of preclinical researchers demonstrated that ECM derived from porcine dermis could be implanted in the rat femoral head, where it facilitated bone regeneration (Ventura et al., 2020). Moreover, ECM derived from the porcine small intestine stimulated osteoporotic bone regeneration in an ovariectomized rat model (Sun et al., 2018a,b).

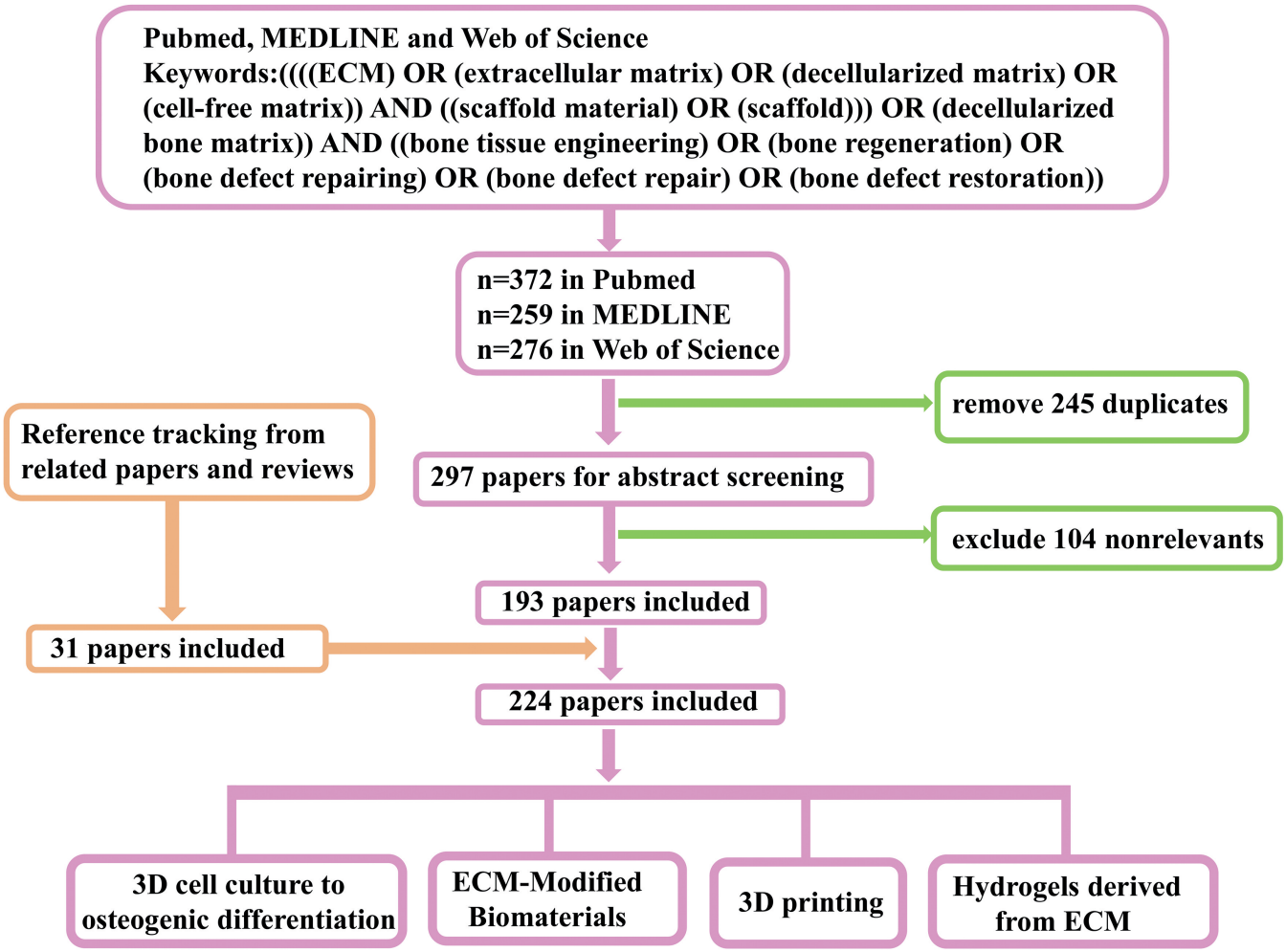

Fig. 1. Flow diagram showing the literature search criteria adopted.

Porcine bone-derived ECM was shown to promote the osteogenic proliferation of human osteoblasts *in vitro* (Obregon-Miano et al., 2020).

Furthermore, xenograft-derived ECM has been applied in clinical treatments. For example, porcine urinary bladder ECM has been used for the treatment of complex wounds in orthopedic trauma patients. Using small intestinal tissue, Li et al. (2017) developed a scaffold that was morphologically similar to natural bone and that could form new bone in calvarial defects.

ECM scaffolds derived from allografts

Human-derived ECM scaffolds are being used more extensively. For instance, whole heart (Zia et al., 2016), ovarian (Hassanpour et al., 2018), pancreas (Sackett et al., 2018), ear (Duisit et al., 2018), tooth (de Sousa Iwamoto et al., 2016), and lung (Gilpin and Wagner, 2018) tissues have been successfully decellularized. Paolo Giuffrida et al. (Giuffrida et al., 2019) used human intestinal ECM scaffolds for 3D cell culture as an

Table 1. Source tissue and species of commercially available biological scaffold materials.

Source species	Source tissue	Product	Application Focus
Porcine	Dermis	Strattice™	Soft tissue
Porcine	Dermis	Avaulta®, CollaMend®	Soft tissue
Porcine	Heart valve	Hancock® II, Mosaic®, Freestyle®	Valve replacement
Porcine	Heart valve	Prima Plus	Valve replacement
Porcine	Small intestine	Oasis®, Surgisis®	Soft tissue
Porcine	Small intestine	FortaFlex®	Soft tissue
Porcine	Small intestine	CorMatrix ECM®	Pericardium, cardiac tissue
Porcine	Urinary bladder	MatriStem®	Soft tissue
Bovine	Pericardium	CopiOs®	Dentistry
Bovine	Pericardium	Perimount®	Valve replacement
Bovine	Pericardium	Lyoplant®	Dura mater
Human	Dermis	Alloderm®	Soft tissue
Human	Dermis	AlloMax™	Soft tissue
Human	Dermis	AlloPatch HD™	Tendon, breast
Human	Dermis	NeoForm™	Breast
Human	Dermis	GraftJacket®	Soft tissue
Human	Dermis	Axis™	Pelvic organ prolapse
Human	Fascia lata	Suspend™	Pelvic organ prolapse
Human	Pericardium	IOPatch™	Opthalmology

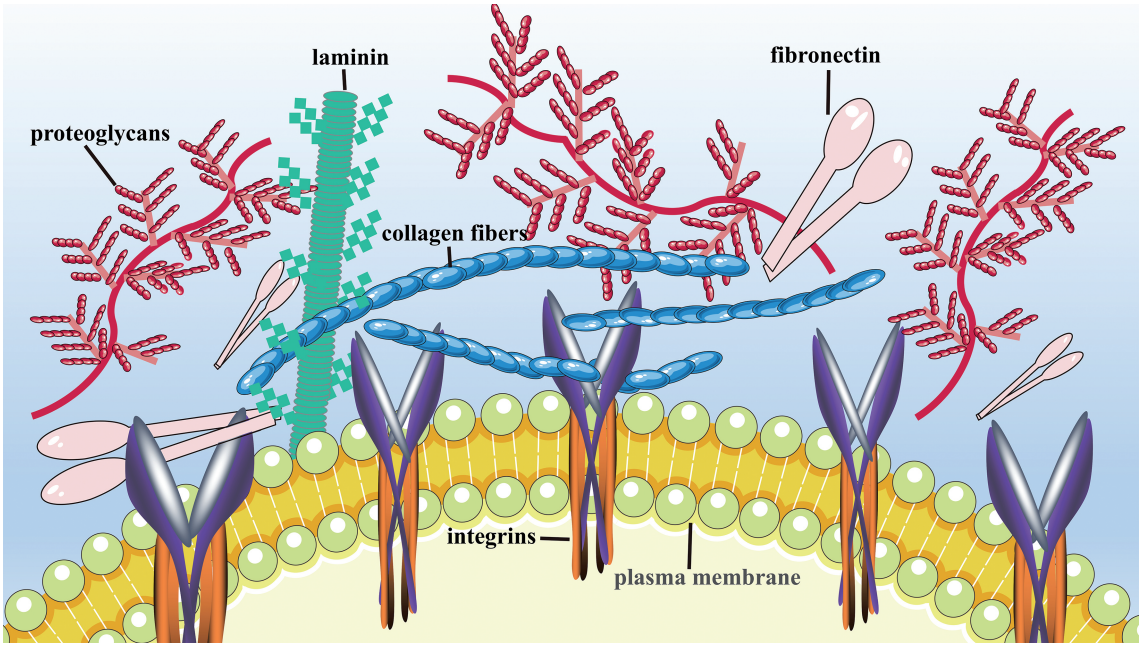

Fig. 2. The main composition of ECM.

innovative platform for disease modeling of human intestinal myofibroblasts. Yu et al. (2020) reported that human nail bed ECM facilitated bone regeneration, and that this effect was mediated by JAK2/STAT3 pathway macrophage polarization. Ye et al. (2000) revealed that human aortic tissue ECM boosted cell attachment in cardiovascular tissue engineering with no potential immunogenic risk.

Likewise, different kinds of ECM from human-derived cells, such as human mesenchymal stem cells (MSCs) (Silva et al., 2020) and human fibroblast/endothelial cells (Junka et al., 2020), were also utilized. Deng et al. (2018) exploited an effective therapeutic approach using adipose stem cell-derived ECM and stromal vascular fraction gel to treat chronic wounds. Al-Abedalla et al. (2015) reported that the success rate of implants of allogenic decellularized bone grafts is similar to that of native bone. This emphasizes the importance of allogenic ECM in bone repair.

Of note, ECM scaffolds can be derived from a single tissue or an entire organ. Currently, clinical products of acellular whole organs have been successfully used in many patients, such as skin transplantation and biological heart valve transplantation (Gilbert, 2012). Organ-derived ECM scaffolds have complex organ geometry and an integrated vascular network system that enhances nutrient supply and is beneficial for regeneration and recellularization. Taken together, allogeneic ECM and xenogeneic ECM have already been used; therefore, an off-the-shelf product for clinical bone regeneration application might be possible. Tissue sources and species of commercially available biological scaffold materials are shown in Table 1.

Fabrication methods of ECM Scaffold

In fact, it is rather difficult to directly fabricate bioscaffolds simulating native ECM because the ECM includes abundant molecules that have not been thoroughly identified. Thus, studies in previous literature usually removed cellular components from tissues or cells to fabricate ECM scaffold (Gilbert et al., 2006; Keane et al., 2012; Manalastas et al., 2020). ECM bioscaffolds usually originate in decellularization of cells, tissues or organs (Crapo et al., 2011). Decellularization is a technique used to produce ECM bioscaffolds by removing cells while maintaining the composition and construction of an ECM (Sart et al., 2020).

An impeccable decellularization should preserve ECM bioactive cues as well as lower the risks of potential transmission of disease. Although there is no quantitative definition about decellularization, the criterion or standard for complete decellularization has

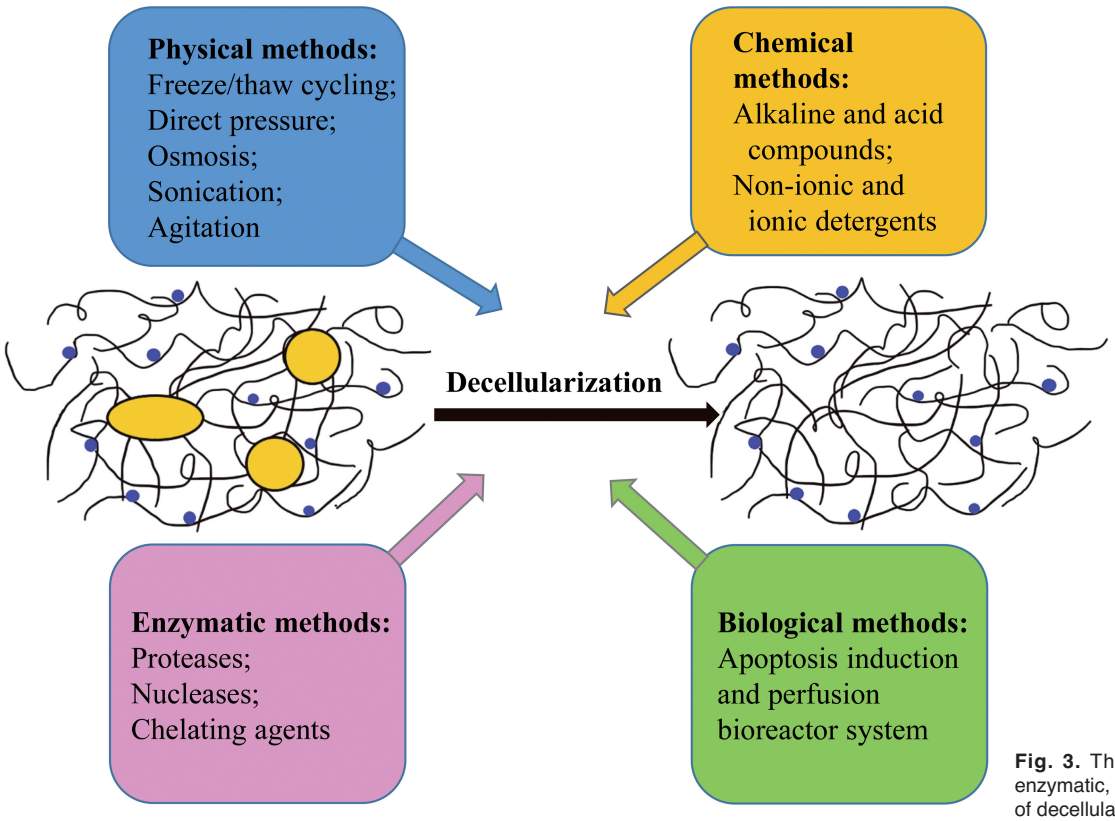

Fig. 3. The chemical, physical, enzymatic, and biological methods of decellularization.

been raised as follows: 1) It has less than 50 nanograms of double-stranded DNA per milligram dry weight of ECM; 2) DNA length is less than 200 base pairs (Nagata et al., 2010; Crapo et al., 2011); 3) Hematoxylin and eosin (H&E) or DAPI (4', 6-diamino-2-phenylindoles) staining show no visible nuclei.

Therefore, all kinds of decellularization techniques have been established to enhance the efficiency of decellularization and can be divided into four categories: physical, chemical, enzymatic, and biological decellularization methods (Fig. 3, Table 2). Actually, the most efficient and powerful decellularization means are a combination of the above four protocols. One representative decellularization protocol typically makes use of physical or chemical methods to disintegrate the cellular membrane, and then dislodge cellular components through enzymatic methods.

Physical methods of decellularization

Physical/mechanical methods, such as freezing/thawing, agitation, sonication, direct pressure, and osmosis are utilized to destroy cellular membranes and lysis cells (Gilbert et al., 2006). Physical methods are able to maximally protect the mechanical properties of the ECM ultrastructure (Gilpin and Yang, 2017).

Nevertheless, physical approaches may cause incomplete elimination of cellular debris. Thus, enzymatic or chemical methods usually need to be supplemented to maintain acellular tissues. For example, in view of compact bone density, Hashimoto et al. Utilized a physical approach of cold isostatic pressure at 30°C for 10 minutes combined with a chemical method of deoxyribonuclease (DNase) treatment at 37°C for 3 weeks for decellularization (Hashimoto et al., 2011).

Chemical methods of decellularization

Various chemicals have been applied in decellularization, containing alkaline and acid compounds, ionic and non-ionic detergents (Somuncu, 2020).

Alkaline compounds can degenerate plasmid deoxyribonucleic acid (DNA) and acids can set DNA apart from the ECM by dissolving cytoplasmic ingredients (Kabirian and Mozafari, 2020). They can lysis cells by disturbing cellular membrane, so, at the same time they can also obliterate collagen and growth factors which will reduce ECM mechanical properties (Wang et al., 2014).

Sodium dodecyl sulfate (SDS) is a frequently-used ionic detergent which can dissolve cellular membranes and nuclei. However, SDS is difficult dislodge from the remaining components. Triton-X is a kind of non-ionic detergent and it can dissolve proteins while preserving enzymatic activity (Gilbert et al., 2006).

Although chemical treatments can effectively remove all cellular components, they can destroy collagen in the remaining ECM (Wang et al., 2014). Hence, to minimize the damage of ECM, chemical approaches should be combined with other methods.

Enzymatic treatments of decellularization

Enzymatic treatments are selected according to the different structures of acellular tissue and generally include: nucleases, proteases (such as trypsin), and chelating agents (ethylenediaminetetraacetic acid (EDTA)) (Crapo et al., 2011).

Especially, EDTA is often utilized with trypsin to remove cell nuclei (Petersen et al., 2010) but may still

Table 2. Source tissue and species of commercially available biological scaffold materials.

Basic technique	Concrete technique	Mechanism and effects on ECM	Reference
Physical Methods	Freezing/ thawing	Intracellular ice crystals disrupt cell membrane; Ice crystal formation can disrupt or fracture ECM.	Brown et al., 2009; Guimaraes et al., 2019
Physical Methods	Agitation	Aggressive agitation lyses cells; Commonly used to facilitate chemical exposure and removal of cellular material.	Tchoukalova et al., 2017
Physical Methods	Direct pressure	Pressure bursts cells; Pressure can disrupt ECM.	Funamoto et al., 2010; Sasaki et al., 2009
Chemical Methods	Acids	Acid solubilizes cytoplasmic components of cells, disrupts nucleic acids; Acid may damage collagen and growth factors.	McCrary et al., 2020; Tebyanian et al., 2019
Chemical Methods	Sodium dodecyl sulfate (SDS)	SDS solubilizes cell and nucleic membranes, tends to denature proteins; SDS removes nuclear remnants and cytoplasmic proteins from dense tissues, but tends to disrupt ultrastructure.	McCrary et al., 2020; Han et al., 2020
Chemical Methods	Triton X-100	Disrupts DNA-protein interactions, lipid-lipid and lipid-protein interactions; Less effective than SDS.	Tebyanian et al., 2019
Enzymatic Methods	Nucleases	Catalyze the hydrolysis of ribonucleotide and deoxyribonucleotide chains; Difficult to remove from the tissue, could invoke an immune response.	Lin et al., 2018; Rahman et al., 2018
Enzymatic Methods	trypsin	Cleaves peptide bonds on the C-side of Arg and Lys; Prolonged exposure can disrupt ECM ultrastructure.	Ramm et al., 2020
Enzymatic Methods	EDTA	Chelating agents bind metallic ions, thereby disrupting cell adhesion to ECM; Typically used with other agents, ineffective when used alone.	Pellegata et al., 2013
Biological Methods	Biological Methods	Activate programmed cell death for decellularization and preserve ECM integrity; An intriguing proposal that needs further investigation.	Raff, 1998

leave cellular remnants (Woods and Gratzer, 2005). To sum up, enzymatic methods eliminate cellular components and retain most collagen components, while enzymatic methods destroy the ECM architecture and tensile strength.

Biological methods of decellularization

Although these above techiniques are effective in decellularization, damage to the remaining ECM still exists. In addition, all of these techniques rely on cell lysis and this leads to an increase in the immunogenicity of cell debris (Boer et al., 2011).

Recently, Bourgine et al. (2014) proposed a biological method to specifically activate apoptosis, a type of programmed cell death, characterized by cell membrane blebbing, cell shrinkage and nuclear fragmentation. Specifically, it can activate programmed cell death for decellularization and preserve ECM integrity. Cells lose track with ECM while cellular components are remained within the apoptotic bodies and cell membranes during apoptosis (Raff, 1998). Cellular contents do not infiltrate into the circumambient matrix in this method, thus averting an unwanted immunoreaction. Apoptosis decellularization is an attractive proposal that needs further research.

All in all, there are no uniform approaches for tissue or cell decellularization. We usually utilize a combination of decellularization approaches to prepare ECM bioscaffolds for bone repair. For example, Abedin et al. (2018) utilized a combination of physical (freeze-thaw), enzymatic (0.25% trypsin, 18 h) and chemical (2.5% SDS, 26 h), methods to decellularize and fabricate bone ECM scaffold.

Applications of ECM scaffolds for bone repair or regeneration osteogenic differentiation promoted by ECM scaffold

Since the first study involving 3D cell culture systems was published in 1968, the number of studies considering the use of 3D culture materials for various applications has increased exponentially (Ravi et al., 2015). The ECM acts as a physical scaffold for cell adhesion and delivery of biochemical and biomechanical signals for cells to initiate migration, differentiation, morphogenesis, and homeostasis. ECM-based scaffolds for 3D cell culture can be divided into decellularized ECM scaffolds and biomaterial-modified ECM scaffolds. As shown in Fig. 4, 3D cell culture can reflect the 3D environment that the cells experience *in vivo* (Castiaux et al., 2019). Hashimoto et al. (2011) found significantly increased alkaline phosphatase (ALP) activity in MSCs seeded on 3D ECM derived from decellularized bone tissue compared with MSCs grown in 2D culture in a tissue culture polystyrene dish.

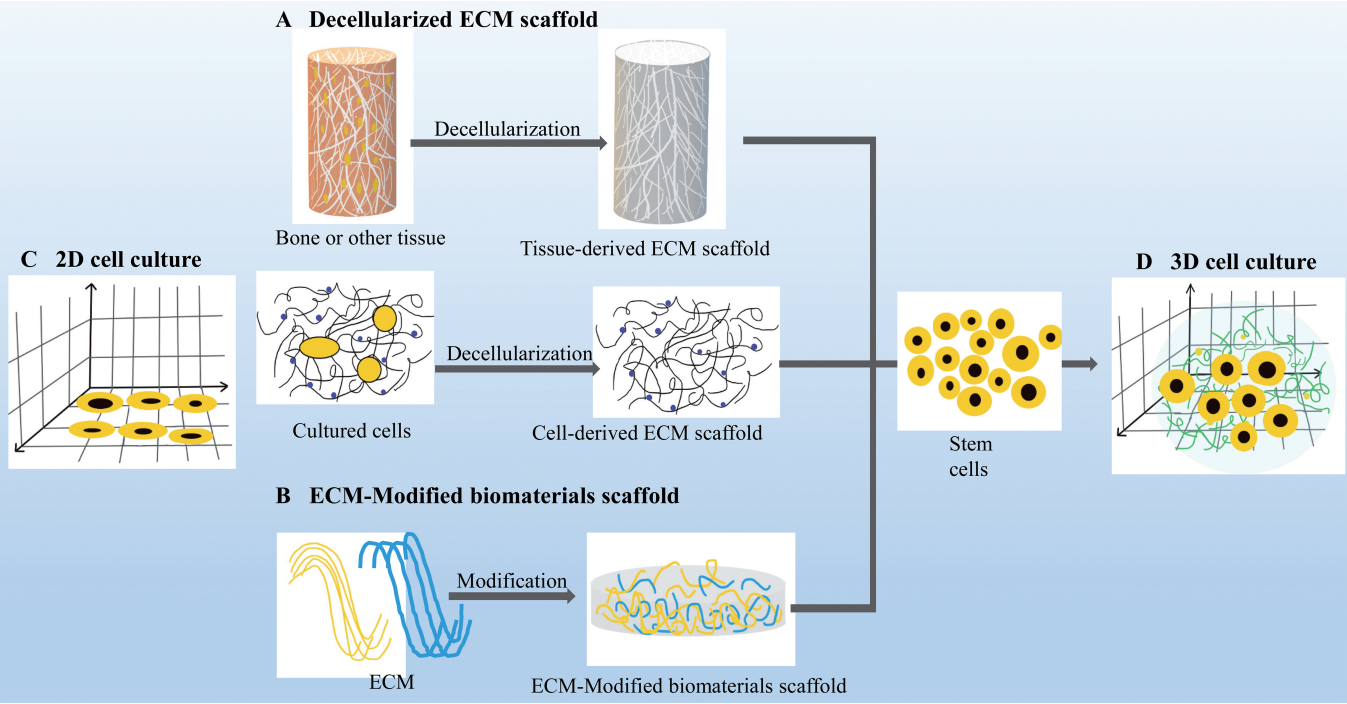

Fig. 4. Schematic preparation of ECM-based scaffold in 3D cellular culture. **A.** decelularized ECM scaffold acquired from issue *in vivo* or cultured cells *in vitro* by decellularization technique. **B.** ECM-modified biomaterials scaffold. Different components and contents of ECM modified with biomaterial-based scaffold. **C.** 2D pattern of cell culture. **D.** 3D pattern of cell culture. Cells are cultured on decelularized ECM scaffold or ECM modified biomaterial-based scaffold to mimic the natural biomaterials.

ECM scaffolds used directly in 3D cell culture

ECM is used extensively in 3D cell culture, with natural ECM being the most widely used (Cukierman et al., 2001; Yamada and Cukierman, 2007). Moreover, Hashimoto et al. (2009) found that the osteogenic differentiation of MSCs in 3D decellularized bone ECM was significantly increased in comparison with cells grown in 2D culture.

ECM derived from different organs or tissues was used to culture cells using 3D approaches and facilitated bone regeneration *in vitro* and *in vivo*. For example, in 2008, Grayson et al. (2008) reported that the osteogenic differentiation of human MSCs was promoted *in vitro* after seeding the cells on decellularized bovine bone ECM. Another study also showed that the osteogenic differentiation of rat MSCs seeded on decellularized bone ECM was promoted *in vitro*, and that cell infiltration with neovascularization appeared *in vivo* in the rat bone defect model. Marolt et al. (Marolt et al., 2012; Rutledge et al., 2014) reported that human embryonic stem cells seeded on bone ECM scaffolds exhibited increased osteogenesis. Hereafter, Fröhlich et al. (2010) created bone constructions *in vitro* utilizing human adipose-derived stem cells (hASCs), decellularized bone ECM scaffolds, and perfusion bioreactors. What is more, the fabricated bone constructs showed viable and compact bone formation. Recently, Yu et al. (2020) discovered that human nail bed ECM facilitated the osteogenic differentiation of bone marrow stem cells, which may be mediated by macrophage polarization via the JAK2/STAT3 pathway, both *in vitro* and *in vivo* using the rat calvarial defect model. Motoike et al. (2019) demonstrated that MSCs mixed with endogenously produced ECM were able to facilitate bone regeneration via direct and indirect osteogenesis. Furthermore, Liu et al. (2019) substantiated that MSCs embedded in their secreted ECM exhibited strong bone formation both *in vitro* and *in vivo* via endochondral ossification. Cunnifffe et al. (2017) reported that MSCs seeded on porcine growth plate ECM scaffolds could accelerate both chondrogenic and osteogenic mineralization *in vitro* and generate endogenous bone regeneration in a critically-sized cranial defect *in vivo*. Their further study showed the potential mechanism was mediated by reduced IL-1β and IL-8 production.

Apart from stem cells, ECM scaffolds can also promote osteogenic differentiation of osteoblasts and other cells. For instance, Reiza et al. (Ventura et al., 2020) found that porcine dermis ECM loaded with biphasic calcium phosphate powder facilitated osteogenic differentiation of MC3T3-E1 cells *in vitro* and improved bone formation *in vivo* in rat cranial defects. Similarly, Xie et al. (2020) reported that small intestinal submucosal ECM scaffolds decorated with poly-lactide-co-glycolic acid promoted osteogenic differentiation of MC3T3-E1 pre-osteoblast cells *in vitro* and enhanced vascularized bone formation *in vivo*.

Modified ECM scaffolds for 3D cell culture

Increasing numbers of studies are focusing on combining ECM with synthetic biomaterials to enhance the textural support and mechanical properties of ECM scaffolds for 3D cell culture. For example, Obregon-Miano et al. (2020) blended pig bone ECM with 20% W/V polyethylene glycol diacrylate to improve the physicochemical properties of the scaffolds, thereby conferring characteristics that are advantageous for bone repair. Chen et al. (2015) coated bone ECM with collagen containing HAP and used the scaffolds for 3D cell culture of rat MSCs. The scaffolds exhibited increased stiffness and enhanced the osteogenic differentiation of rat MSCs *in vitro*. Junka et al. (2020) coated PCL with fibroblast/endothelial cell-derived ECM and confirmed that this bioactive scaffold significantly enhanced osteoblast attachment and osteogenic differentiation *in vitro*. Chai et al. (2017) loaded ECM derived from human periosteum-derived osteoprogenitor cells on titanium (Ti)-based scaffolds and showed that the scaffolds guided bone regeneration in a critical-sized rat calvarial defect.

Table 3. ECM Sources and applications in 3D cell culture.

ECM sources	Cells used *in vitro* experiments	Animals used *in vivo* experiments	Reference
bovine bone	hMSCs	----	Grayson et al., 2008
porcine bone	rMSCs	subcutaneous implantation of rat	Hashimoto et al., 2011
porcine bone	hESCs	munodeficient (SCID-beige) mice	Marolt et al., 2012
bovine bone	hASCs	----	Frohlich et al., 2010
human nail bed	BMSCs	calvarial defect of rat	Yu et al., 2020
hMSCs	hMSCs	----	Motoike et al., 2019
porcin growth plate	hMSCs	cranial defect of rat	Cunniffe et al., 2017
porcine dermis	MC3T3-E1	cranial defect of rat	Ventura et al., 2020
SIS	MC3T3-E1	subcutaneous implantation of rat	Xie et al., 2020

hMSCs, human mesenchymal stem cells; rMSCs, rat mesenchymal stem cells; hESCs, human embryonic stem cells; hASCs, human adipose-derived stem cells; SIS, small intestinal submucosa.

Osteogenic differentiation of MSCs promoted by modification of the ECM microenvironment

In 2019, Fu et al. (2019) created a bismuth sulfide/HAP film to promote MSC osteogenic differentiation *in vitro* by tuning the *in vivo* photoelectric ECM microenvironment, which is mediated by the Wnt/Ca^{2+} signaling pathway. Interestingly, this suggests that by changing the ECM microenvironment, we can stimulate and control cell fate and improve bone regeneration in a noninvasive manner.

Moreover, Chen et al. (2016) found that decullarized ECM scaffolds with different stiffness but same microstructure were able to promote MSC osteogenic differentiation and bone regeneration in large bone defects of rabbit. Their findings suggested that simply changing the ECM scaffold stiffness can be one of the most intriguing strategies for accelerating bone healing.

ECM-modified biomaterials and improved osteogenesis

As mentioned previously, ECM scaffolds have exhibited favorable biological activities; nevertheless, their mechanical performance is inadequate for bone tissue support and regeneration (Hashimoto et al., 2011). Hence, synthetic biomaterials, including PCL, HAP, and Ti, are typically combined with cell- or tissue-derived ECM to improve the mechanical properties and to further enhance cell–material interactions.

ECM-modified PCL

PCL is an FDA-approved synthetic material that has excellent mechanical properties. It is biodegradable and biocompatible and is extensively used in biomedical applications (Jeon and Kim, 2014; Saderi et al., 2018). For example, Carvalho et al. (2019) used PCL electrospun microfibrous scaffolds to load cell-derived ECM and found that these scaffolds significantly promoted cell proliferation and increased osteogenic gene expression levels. Likewise, Thibault et al. (2010) reported that MSCs seeded onto PCL/ECM constructs enhanced the osteogenic differentiation of MSCs. Shekaran et al. (2016) also found that ECM-coated PCL microcarriers promoted the proliferation of MSCs and bone formation *in vivo*.

ECM-modified HAP

Apart from PCL, HAP has also been used in bone engineering to improve mechanical properties, bioactivity, and cell growth (Zhang et al., 2008). HAP scaffolds have been used extensively for bone repair, largely because they mimic the chemical composition and physical structures of natural bone ECM (Kareem and Tanner 2020). HAP was also widely used in combination with ECM. For instance, Tour et al. (2011) reported that HAP scaffolds combined with ECM derived from rat tissues enhanced the osteogenic effect of the HAP microparticles. Dennis et al. (2017) found that the combination of ECM derived from demineralized bone or cartilage, with hyaluronic acid and HAP nanoparticles, improved cell viability. Furthermore, Chen et al. (2016) coated rabbit bone ECM with HAP and demonstrated that scaffolds with the appropriate stiffness increased bone regeneration. Interestingly, the authors further showed that the mechanism of matrix stiffness influencing bone regeneration was driven by stromal cell-derived factor-1a, which mediated MSC migration (Chen et al., 2016).

ECM-modified other biomaterials

Ti is a widely used metal in biomedical applications because of its biocompatibility. Datta et al. (2005) cultured MSCs on a scaffold of ECM combined with Ti fibers and found that the synthetic bone-like ECM enhanced the osteoblastic differentiation of MSCs *in vitro*. Also, da Costa Fernandes et al. (2018) found that Ti-decorated ECM augmented pre-osteoblast viability and proliferative ability. Gibson et al. (2014) incorporated decellularized ECM from different tissues into nanofiber scaffolds. Interestingly, scaffolds containing decellularized bone, cartilage, and fat ECM facilitated hASC osteogenesis, while spleen and lung ECM reduced hASC osteogenesis.

ECM used in 3D printing to produce customized scaffolds for bone repair

3D printing of ECM scaffolds has recently emerged (Da Silva et al., 2020). 3D bioprinting can create complex and customized structures by integrating cells and biomaterials (Gopinathan and Noh, 2018; Dzobo et al., 2019). Recently, ECM has been used in 3D printing more extensively to produce patient-tailored or customized scaffolds for bone repair in bone tissue engineering.

3D printing of ECM scaffolds

De Peppo et al. (2013) reported that induced pluripotent stem cells seeded on engineered 3D printed ECM exhibited strong osteogenic potential both *in vitro* and *in vivo*. Jung et al. (2018) developed a bioink using cartilage ECM and silk fibroin for 3D printing of irregularly shaped cartilage, which was made feasible because the viscosity of the bioink can be easily controlled. Furthermore, Chen et al. (2019) used autogenous bone ECM to customize a 3D printing program for skull tissue engineering. Further study proved that the customized autogenous implants were able to promote bone mineralization and produce vascularized bone. Furthermore, to improve the mechanical performances of 3D printing ECM scaffolds, Zhao et al. (2020) developed a high-viscosity slurry ECM bioink, which significantly improved the 3D cell

printing performance and induced regeneration of pure acellular matrix bioink.

Requirement of additional materials for 3D printing of ECM scaffolds

The mechanical properties of bone ECM make it difficult to print 3D scaffolds. Therefore, for 3D printing, additional materials are usually combined with the ECM to improve the mechanical properties of the resulting bone scaffold (Freeman et al., 2019). For instance, Freeman et al. (2019) used functionalized PCL with decellularized bone ECM to produce osteoinductive filaments for 3D printing. This modification improved the mechanical properties of the scaffold and improved cellular attachment and osteogenesis of MSCs. Silva et al. (2020) decorated PCL scaffolds with MSC-derived ECM and generated structurally well-defined bioactive scaffolds. The cell-derived ECM within the PCL scaffolds prominently facilitated the attachment and proliferation of MSCs. Rentsch et al. (2014) reported that ECM embroidered 3D printing PCL scaffolds enhanced calvaria bone regeneration. In addition, Fahimipour et al. (2019) encapsulated MSCs in 3D printed bone ECM combined with bone morphogenic protein 2. These constructs promoted the osteogenic differentiation of MSCs *in vitro* and induced bone formation in a rat cranial defects model.

3D bioprinting with the ability to print cell-laden ECM scaffolds is a promising method for bone repair. However, the mechanical properties of bioprinted scaffolds are not ideal, and this constitutes one defect of this technique. Therefore, more effort should be invested in developing ECM-derived 3D printing bioink with higher mechanical capacities.

Use of hydrogels derived from ECM for bone repair

Evidence has proven that the success or failure of biomaterial scaffolds in various applications is contingent on the host immune reaction. Therefore, many studies have focused on using ECM gel rather than ECM parcel due to the lower immunogenicity of hydrogels (Wu et al., 2019). Hydrogel is an ideal scaffold material for bone tissue engineering due to its ECM-like features, such as favorable softness, porosity, and aquosity (Jiang et al., 2019). Another major advantage of acellular ECM-based gel is its favorable plasticity; thus, it can fill and repair random-shaped defects (Petrou et al., 2020). ECM hydrogels have been used in many preclinical studies to repair injuries incurred by, for example, a stroke (Ghuman et al., 2016), optic nerve damage (Ren et al., 2018), and ulcerative colitis (Keane et al., 2017).

As for bone repair, in 2014, Smith et al. (2014) found that bone-derived ECM hydrogel scaffolds facilitated skeletal tissue formation in chick femoral defects. Hereafter, Alom et al. (2018) demonstrated that hydrogels sourced from bovine bone ECM stimulated the osteogenic differentiation of C2C12 myoblasts and mouse primary calvarial cells, even without the use of osteogenic medium. Recently, Shridhar et al. (2019) discovered that trabecular bone ECM-derived hydrogel augmented osteogenic differentiation of hASCs. Taken together, ECM hydrogels are perfectly suited for bone repair in bone tissue engineering and clinical applications.

The mechanisms for ECM in enhancing bone repair

Bioactive ingredients play an important role in bone regeneration

As described above, ECM is composed of different kinds of bioactive ingredients, such as fibronectin, collagen fibers, and proteoglycans. Fibronectin has an inhibitory effect on osteoclasts, possible mechanism associated with inhibition of NF-κB signaling pathway and reduction of intracellular reactive oxygen species (ROS) (Li et al., 2018). Type I collagen can increase the level of antioxidant enzymes in mesenchymal cells and enhance the resistance of mesenchymal stem cells to premature cell senility induced by oxidative stress by activating the SIRT1-dependent signaling pathway (Zhou et al., 2018). In addition, glycosaminoglycans promote osteoblast differentiation and inhibit osteoclast differentiation by up-regulating the expression of OPG (Salbach-Hirsch et al., 2014).

Physical arrangement and surface morphology of bioactive molecules regulate the behavior of stem cells to promote bone regeneration

Fibronectin derived from chondrocyte ECM is compact and orderly arranged. The pyknotic and ordered arrangement of these specific proteins enables the fibroblastogenic ECM to significantly upregulate the osteogenic related proteins: alkaline phosphatase, osteopontin, osteocalcin and type I collagen, thus exerting the osteogenic biological effects of ECM (Bae et al., 2012). Changing the elastic modulus and surface morphology of ECM regulates stem cell behavior by activating the FAK/RhoA/ROCK/MAPK signaling axis (Janson and Putnam, 2015). The complex mechanism of ECM surface micromorphology regulating osteogenic differentiation may also involve two protein members in the Hippo signaling pathway: YAP and TAZ. The response of cells to the nanotopology of DECM is related to YAP and Taz (Mosqueira et al., 2014). TAP and TAZ exert a combined effect to promote osteogenesis by regulating of osteoblast activity, matrix quality, and osteoclastic remodeling (Kegelman et al., 2018). At present, there are few reports on the relationship between DECM and TAP /TAZ, and the related mechanism needs to be further studied.

ECM contains large amounts of endogenous growth factors

ECM provides a microenvironment rich in endogenous growth factors for bone regeneration and

continues to slowly release growth factors such as IGF-1to promote proliferation and osteogenic differentiation of seed cells (Wei et al., 2017). Moreover, ECM with three-dimensional spatial structure composed of bioactive molecules may recruit and release a large number of growth factors.

Integrin-mediated communication between cells and ECM regulates cell behavior

Integrins cooperate to promote epithelial cell adhesion and growth (Yazlovitskaya et al., 2019). They play a bidirectional role in information transmission between cells and ECM. Not only that, but integrins also play an important role in the initial adhesion of cells to biomaterial surfaces (Rahmany and Van Dyke, 2013). Integrin can regulate cell differentiation through the Wnt signaling pathway, and Wnt5a can increase the expression of integrin mRNA and protein and further regulate osteogenic differentiation of mesenchymal stem cells (Olivares-Navarrete et al., 2011). ECM also regulates the osteogenic differentiation of mesenchymal stem cells through Integrin α5, Akt, and GSK-3β pathways (Sun et al., 2018a,b). In short, the mechanism of ECM in bone regeneration is extremely complex and diverse. Only by digging and understanding the mechanism of ECM in bone regeneration and further optimizing the biocompatible materials of ECM, can the transformation from basic research to clinical application be realized.

Future perspectives and current challenges

Advantages of using ECM scaffolds in repairing bone defects

ECM scaffold materials have many advantages. 1) The 3D structure of the ECM material source is intact. In tissues, cells are surrounded by the ECM, a complex 3D structure that contains many types of collagen and bioactive factors. The retention of this structure is critical for bone repair. 2) The ECM scaffold retains the original components and growth factors necessary for cell adhesion, proliferation, and differentiation, thereby retaining the original natural microenvironment to facilitate the growth and repair of new tissues. For bone, some important growth factors, including TGF-1, FGF, and EGF, the retention of which has special benefits for bone regeneration, can enhance cell function and promote vascularization. 3) ECM has low immunogenicity. In the process of decellularization, the cell membrane and its surface receptors are effectively cleared; therefore, *in vivo* transplantation hardly causes any immunogenicity, and ECM scaffolds can be effectively used for allotransplantation. 4) ECM scaffolds are biodegradable. The ECM scaffold can degrade naturally, and its polymer chain can be digested into small molecules, which eventually get absorbed and metabolized by the body. The degradation products of ECM scaffolds do not affect normal cell proliferation. 5) ECM scaffolds are biocompatible. The ECM scaffold itself and its degradation products have no toxic or side effects on the body, and they can reduce the occurrence of autoimmune reactions such as inflammation. The ECM provides a favorable scaffold for seed cells and for molecules related to bone regeneration. Furthermore, ECM scaffolds provide a 3D structure and a favorable growth environment for seed cells, which can enter the lacunae cavities left by the decellularization process, and allow the cells to interact with the ECM components to produce new bone.

Decellularized ECM is easy to produce and can be set up to generate decellularized bone matrix of different shapes, sizes, and hardness. Bone marrow- or periosteum-derived seed cells from the patient can be directly implanted at the injury site or seeded in the scaffolds and allowed to grow and differentiate *in vitro* before surgically replanting in the patient's injured bone. Other advantages of using ECM as a scaffold material include its relatively low cost and low risk of spreading infectious diseases.

Challenges of using ECM scaffolds in repairing bone defects

Although there are many advantages to using ECM scaffolds in repairing bone defects, there are some challenges that need to be addressed. These include 1) optimizing the preparation process to minimize damage to the ECM, 2) determining the optimum composition of seed cells and growth factors to produce the best repair effect, 3) further study of the immunogenicity and teratogenicity of allogeneic or heterogenic ECM, and 4) ensuring that ECM scaffold materials constructed *in vitro* meet the clinical application standards. It is also necessary to standardize the safety evaluation and quality control standards for clinical application of the product.

Concluding remarks

The ECM is an effective scaffold material. It is highly biocompatible and can be combined with cells and molecules that are essential for bone regeneration. With the rapid development and progress in acellular bone matrix generation and bone tissue engineering, ECM will have broad application prospects as a scaffold material for bone regeneration and reconstruction needed for the treatment of bone defects, fractures, and other bone diseases.

Acknowledgements. This work was supported by grants from the National Natural Science Foundation of China (No. 81700935), and CAMS Innovation Fund for Medical Sciences (2019-I2M-5-038).

Conflict of Interest. The authors report no conflicts of interest in this work.

Statement. This article does not contain any studies with human participants performed by any of the authors.

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Accepted September 2, 2021