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REVIEW

Current advances for bone regeneration based on tissue engineering strategies

Rui Shi[1,*], Yuelong Huang[2,*], Chi Ma[1], Chengai Wu[1], Wei Tian (✉)[1,2]

[1]*Institute of Traumatology and Orthopaedics, Beijing Laboratory of Biomedical Materials, Beijing Jishuitan Hospital, Beijing 100035, China;* [2]*Department of Spine Surgery of Beijing Jishuitan Hospital, The Fourth Clinical Medical College of Peking University, Beijing 100035, China*

Abstract **Bone tissue engineering (BTE) is a rapidly developing strategy for repairing critical-sized bone defects to address the unmet need for bone augmentation and skeletal repair. Effective therapies for bone regeneration primarily require the coordinated combination of innovative scaffolds, seed cells, and biological factors. However, current techniques in bone tissue engineering have not yet reached valid translation into clinical applications because of several limitations, such as weaker osteogenic differentiation, inadequate vascularization of scaffolds, and inefficient growth factor delivery. Therefore, further standardized protocols and innovative measures are required to overcome these shortcomings and facilitate the clinical application of these techniques to enhance bone regeneration. Given the deficiency of comprehensive studies in the development in BTE, our review systematically introduces the new types of biomimetic and bifunctional scaffolds. We describe the cell sources, biology of seed cells, growth factors, vascular development, and the interactions of relevant molecules. Furthermore, we discuss the challenges and perspectives that may propel the direction of future clinical delivery in bone regeneration.**

Keywords bone tissue engineering; stem cell; bone scaffold; growth factor; bone regeneration

Introduction

Bone tissues exhibit an excellent ability to spontaneously regenerate and repair themselves from the surrounding osteoprogenitor cells without scarring. However, spontaneous repair may not occur in patients with bone injuries beyond the extent of self-healing. This characteristic may lead to non-union, scar formation, and even long-term persistent bone defects. Prevalent therapeutic strategies for bone regeneration mainly involve autologous, homologous, and xenologous bone grafting. The limitations of available clinical applications of these graftings are caused by donor site morbidity and autogenous bone deficiency in autografts, infectious risks, and rejection [1,2]. Hence, bone graft substitutes or novel approaches have long been explored to treat large bone defects.

Bone tissue engineering (BTE) is an alternative technique that requires biology, engineering, and material science to create biological substitutes to reduce the known drawbacks of traditional grafts. BTE mainly focuses on skeletal stem cells, biological growth factors, and biocompatible scaffolds to enhance bone formation and repair (Fig. 1). Meanwhile, the establishment of sufficient vascular system is crucial to satisfy the nutrient supplement and removal of waste during bone tissue regeneration.

The BTE scaffold should closely mimic the natural bone extracellular matrix (ECM) that provides basic structure and correct microenvironment for bone tissue growth. Among the different types of third generation scaffolds, several essential features are required, such as the biomimetic 3D structure, excellent biocompatibility, adaptable biodegradability, pre-vascularized structure, osteoconductivity, and less immunogenic responses [3–5]. To design these biological functionalized scaffolds, several novel scaffold fabrication approaches will be discussed in this review.

The accessibility of seed cells is a primary concern in BTE, and stem cells are traditionally the most important source for the seed cells in BTE. Acting as a seed cell, the stem cells can be produced in large quantities through relatively simple harvesting procedures, have a stronger

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Correspondence: Wei Tian, tianweijst@vip.163.com

*Co-first authors: These authors have contributed equally to this work.

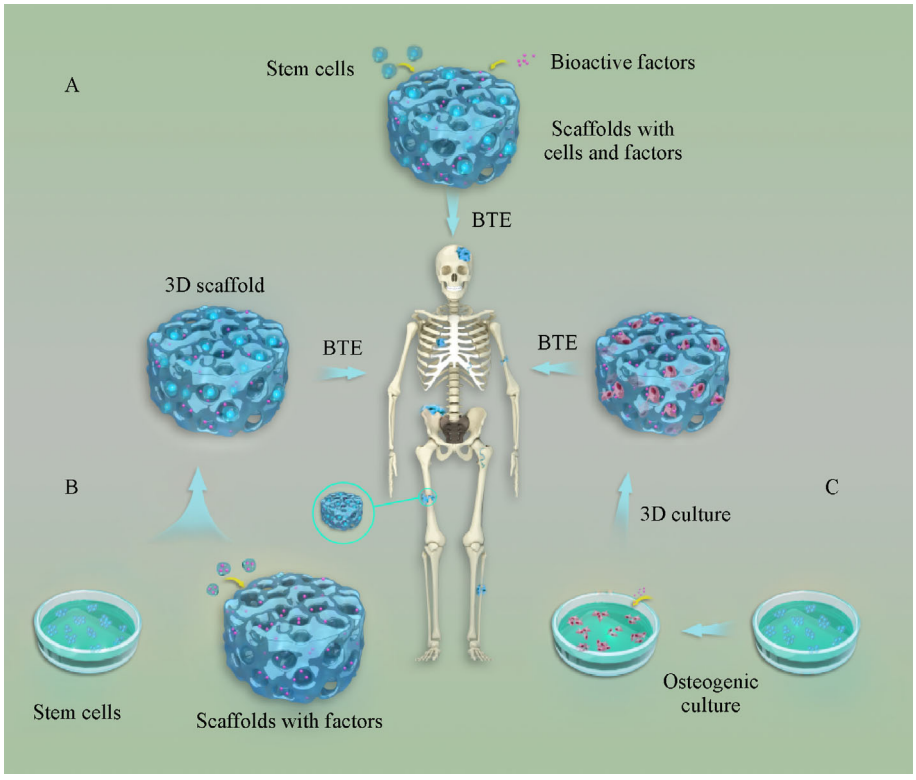

Fig. 1 Main methods and essential procedures that compose bone tissue engineering (BTE). The BTE involves stem cells, biological growth factors, and biocompatible scaffolds that are transplanted to the bone defect area. Three different patterns are used in BTE as follows: (A) cells and factors directly blended with scaffolds; (B) scaffolds combined with factors first and then cocultured with stem cells; and (C) osteogenic culture and 3D culture with scaffolds and factors.

osteogenic differentiation potential, and importantly have less ethical controversy surrounding them [6,7]. However, different types of cells have respective limitations and drawbacks, and recent studies partly concentrate upon finding feasible stem cells to fully develop the potential of BTE.

Bioactive factors belong to a large class of cytokines that stimulate cell growth, migration, and differentiation and play important roles in bone tissue regeneration [8]. The induction of signaling cascades by various growth factors, including osteogenesis and angiogenesis, may provide specific regulations in the bone regenerative process. Delivery systems based on different biological scaffolds have been described in the literature to control small osteoinductive molecules [9]. Although many challenges and limitations remain in tissue regeneration, such as immune rejection and clinical applicability, current studies have achieved major advances and show potential in combating these problems.

Numerous reviews have reported separately on the three key elements in BTE [1,2,4–6,9], but none of these reviews comprehensively described the scaffolds, seed cells, biological factors, and their interconnecting relationships. Therefore, the present review will systematically discuss the current developments in bone regeneration based on tissue engineering, particularly in terms of skeletal stem cells, bioactive factors, vascular formation, and advantageous scaffold fabrication. In addition, the interactions among cells, growth factors, and scaffolds are also reviewed to further understand the mechanism of action in regenerative strategies. The current advances in the BTE field reviewed in this article will help solve the translational issues from preclinical *in vivo* models to clinical delivery.

BTE scaffolds

Tissue engineering scaffolds provide the extracellular microenvironment for the “replace and trigger” mechanism of tissue regeneration [5]. The ideal BTE scaffold should be manufactured from biomaterials that imitate the natural structure and properties of bone ECM [10], including, but not limited to, providing temporary mechanical support, acting as a substrate, encouraging bone cell migration, supporting and promoting osteogenic differentiation, enhancing cellular activity toward scaffold-host tissue integration, and controlled degradation [11].

Classification of the bone scaffolds

The scaffolds for BTE can be classified in various ways. Classification based on material composition, i.e., metals, ceramics, polymers as well as their composites, and the morphology that the system adopts are all considered. The scaffolds can be divided into solid support structures with interconnected pore networks and hydrogels containing encapsulated cells.

Three generations are defined according to the evolution of bone implant devices. First-generation substitutes refer to the inertness with the tissue microenvironment. Second-generation scaffolds have bioactive interfaces which avoid the formation of fibrous layers and improve osteointegration. Third-generation scaffolds try to mimic autografts by utilizing biomaterials capable of inducing specific cellular responses at the molecular level, and this generation also includes the bioactivity and biodegradability of second-generation devices [10].

Physical characteristics of the scaffold, such as porosity, pore geometry, interconnectivity, and surface topography, offer the transmission of nutrient substances and cell adhesion that play a crucial role in tissue regeneration [4]. Meanwhile, the scaffold provides the support as the ECM that scaffold stiffness and mechanical resistance are important properties. In addition, the scaffolds' desirable feature may be controlled degradation after they are implanted to get void space for new tissue growth [10]. The mechanical properties and degradation of the scaffold depend on the material properties and the porosity geometry of its structure that should be similar to the properties of the replaced bone tissue to prevent stress shielding. The degradation rate should be as close to the tissue growth rate to maintain stable mechanical properties in the tissue-scaffold compound during regeneration.

A mass of biomaterials are being explored to incorporate physical, chemical, and biological signaling paths to create appropriate regenerative microenvironments. These scaffolds may contain the following materials: (1) bioceramics — hydroxyapatite (HA) or calcium phosphates that typically exhibit good bone inductivity and display high compressive strength; (2) synthetic polymers— a variety of macromolecules with favorable biodegradability to create materials for tissue engineering; (3) natural polymers, such as collagens, fibrin, and hyaluronic acid, which are intrinsically biocompatible and have reached clinical application with slight immune reaction; and (4) hydrogel–hydrated polymer chains, which provide active potential in the delivery of cells and growth factors. These materials can typically support the adhesion of cells and other ECM proteins, enable migration of cells, and facilitate incorporation of bioactive molecules and nutrients and their subsequent control, in time and space, targeted release [3,12].

Recent development of bone scaffolds

Over the past several decades, several key issues of BTE have not been resolved completely by tissue engineers. These issues include vascularization and tissue necrosis [13]. Thus, numerous studies have suggested that the success of BTE strategies critically depend on the ability to mimic the natural physiology of the patient and the recreation of the blood vessel network within the implanted scaffolds. Insufficient vascularization results in the formation of a fibrous capsule around the scaffolds and limits the transport of nutrients and removal of waste [14]. Therefore, major effort has been exerted to understand the chemical markers that regulate cellular processes to design 3D scaffolds that have synergistic osteogenic and angiogenic properties. Several scaffold fabrication approaches have emerged in recent years. This section mainly focuses on some of the more advanced and popular strategies in this field, including scaffolds with biomimetic composites and 3D structure, biological functionalized scaffolds, and pre-vascularized structures within the scaffolds. These technologies have been applied both individually as well as cooperatively.

Biomimetic BTE scaffolds

Bone extracellular tissues can be understood as a nanocomposite 3D scaffold composed of organic (predominantly collagen-type I) and inorganic (nanocrystalline HA) components. The collagen fibrils include a 67 nm periodicity and 40 nm gaps between collagen molecules. The HA crystals are embedded in these gaps between the collagen molecules and increase the rigidity of the bone [14]. Furthermore, the bone is a highly hierarchical and specialized tissue with special pore structures, cellular organization, and material composition that direct its function. The bone microporosity allows cell migration and vascularization, while nano- and micrometer scale features facilitate cell adhesion, differentiation, and mineral nucleation. Therefore, fabrication of scaffolds which mimic the material composition and 3D structure of the extracellular bone environment will certainly be a focus of next-generation orthopedic constructs [15].

Biomimetic of composition and microstructure

The bone tissue represents a biological nanocomposite. Several nanomaterials, such as nanofibers, nanoparticles, nanotubes, and hydrogels, have recently emerged as promising candidates to fabricate bone scaffolds to better mimic the natural bone ECM in micro- and nanostructure. These biomimetic efforts include the selection of biomaterials present in the native bone (mainly HA and collagen)

and fabrication of scaffolds bearing multiple-scale architectures with different nanoscale components.

The most important methodology employed in the fabrication of biomimetic bone nanocomposites is molecular self-assembly [16–18]. These self-assembled biocomposites have different structural features from nano- to microscale. Composites and nanoscale structural features have generally been shown to possess regulatory effects on osteoblasts and stem-cell behavior over multiple aspects. Recent studies have demonstrated that nanocomposites with biomimetic bone compositions represent good platforms which can recapitulate the regeneration of natural bone ECM through osteo-mimetic architectures [16,17]. Meanwhile, scaffold pore structure, such as size, shape, volume, and interconnectedness, is a key consideration for cell proliferation, migration, differentiation, vascularization, and better spatial organization for cell growth and ECM formation [19–21].

A great number of conventional manufacturing techniques, such as solvent casting combined with particle leaching, fiber fabrication, membrane lamination, freeze drying, and gas foaming, have been developed to fabricate porous scaffolds. However, the shape, pore size, geometry, and interconnectivity cannot be precisely controlled by these techniques. Therefore, patient-specific scaffolds cannot be adapted for large-scale automated fabrication.

The most significant advantage of additive manufacturing (AM) over the aforementioned conventional methods is the former's production of more precise, intricate, and patient-specific scaffolds with exact control on the porous architecture [22,23]. AM technologies have been developed to produce 3D porous interconnected scaffolds using computer-aided design software by controlling their total architecture. A series of AM techniques, including 3D printing (3DP), stereolithography (SLA), fused deposition modeling (FDM), selective laser sintering (SLS) and biofabrication/bioprinting, have been developed to construct BTE scaffolds [24]. Bioprinting is a specialized AM technology and cell-laden method which will be introduced in further section.

3DP: The 3DP technology is a power-binding-based method. A layer of powder is laid on the building platform, and a liquid binder is then subsequently sprayed onto the surface of the powder layer to bind the powder granules following the pattern of a 2D image of the first cross-section. After one layer is completed, a second is laid over the first layer. This process is repeated, until all the layers have been printed. This procedure is followed by depowdering and sintering processes. Numerous researchers are convinced that 3DP can be adapted to produce porous ceramic-based bone scaffolds [25]. This method has been validated in several *in vivo* studies (Table 1). Thus, investigators are working on acquiring a number of clinical applications in the near future.

SLS: SLS is also a powder-based AM technique that uses a high-powered laser to melt powder for structural construction. The SLS method can create complex structures with a relatively wide range of powder materials, such as poly (lactic acid) (PLA), polycaprolactone (PCL), and bio-ceramics. A number of SLA scaffolds, including nano-HA/PCL and PCL scaffolds, have been implanted in rabbit femur defects, and these scaffolds have shown good biocompatibility and clear bone defect healing [26]. Although the potential of scaffolds fabricated via SLA for BTE has been recognized, this technique is limited by the high operating temperatures required during manufacturing. This high temperature has been demonstrated on numerous occasions to cause a change or damage in the composition of the materials used [27].

SLA: SLA is a photosensitivity-based AM technology and works through exposure of a liquid photocuring polymer to UV radiation and one-by-one stacking of the consolidated monolayers. Several materials showing good biocompatibility and quick photo-polymerization abilities, such as gelatin, polypropylene fumarate (PPF) and

Table 1 Additive manufacturing (AM) techniques for the production of cell-free bone tissue engineering (BTE) scaffolds

AM technologies	Printing materials	Main achievements in BTE	References
Powder-based: 3DP SLS SLM/SEBM	Powder of ceramic, mental, polymer, and their composites	Several *in vivo* studies have demonstrated its validity and potential in clinical practice	[26, 29–35]
Photosensitivity-based: SLA	Polymers, hydrogels with good photopolymerization capabilities (PPF, gelatin, and TCM-based)	PDLLA composites and PPF/ diethyl fumarate scaffolds were fabricated and cultured with hMSC and MC3T3-E1 osteoblasts	[36–38]
Melt-extrusion-based: FDM	Biodegradable polymers and their composites (PCL, PLGA, PDL, PCL/ tricalcium phosphate, PLGA/TCP/HA)	PCL composite-based scaffolds were fabricated and tested *in vitro*; PCL/HA bones enhanced the new bone formation in long load-bearing goat femur bone segmental defect model	[39–42]

trimethylene carbonate (TMC), have been actively studied for BTE over the past few years. However, the major limitation of SLS is the use of photocurable resins, which are not approved by the US FDA for *in vivo* applications [28].

Selective laser melting (SLM): The SLM is one of the emerging advanced additive manufacturing technologies. This method provides an ideal platform for producing titanium components. The SLM first is started with the creation of a 3D model followed by mathematical slicing of thin layers. These layers are transferred to a specific SLM device to realize the final product layer by layer. SLM provides numerous advantages, such as net shape ability for complex shapes, high material utilization, lower production time, and minimal machining. Hence, SLM is an attractive option to produce titanium components, especially with complex shape [29].

Selective electron beam melting (SEBM): The SEBM is a powder-bed-based AM technology. Although this process resembles the more common SLM process, in SEBM, a laser beam, instead of an electron beam, is used as heat source. Both processes are powder bed-based layer-by-layer techniques in which a powder layer is applied on a building area and selectively molten by a moving heat source. The main advantage of SEBM is the high-power electron beam which can be moved at extremely high velocities and can also be used for heating. SEBM is especially suited for high-performance materials which are otherwise difficult or impossible to process [30].

Deposition modeling (FDM): The FDM utilizes thermoplastic polymer fibers which are heated and extruded out of a moving nozzle layer-by-layer. Several biodegradable materials, such as PCL, poly (D, L-lactide-glycolide) (PLGA) and poly (D, L-lactide) (PDL), have been used for deposition. A series of PCL-based composites, such as PCL/tricalcium phosphate (TCP) and PCL/HA, have also been investigated. The new bone formation ability of FDM-fabricated PCL/HA artificial grafts were also investigated in long load-bearing goat femur bone segmental defect models and canine partial sternal defect model *in vivo* (Table 1).

In the above-mentioned AM technologies, powder-based 3DP is considered the most suitable for constructing biomimetic BTE scaffolds, because this method can achieve favorable structures and use a variety of materials. The calcium phosphate (CaP) powder for 3D-printed bone scaffolds is considered as a particularly interesting solution for bone regeneration [24].

Although the above-mentioned biomimetic strategies have been utilized to prepare biomimetic scaffolds from nano- to macrostructures, the combination of molecular self-assembly and AM technologies to form scaffolds is still an under-explored area. The rational design of biomimetic materials requires incorporation of a multi-level design covering many length scales. The challenge to BTE is probably the use of a collection of technologies in combination with nanomaterials to form multi-dimensional architectures.

Biological functionalized scaffolds

Multiple and sequential signaling molecule delivery

The biochemical cues which should be used in BTE scaffolds have been inspired by the signals naturally presented in materials within the bone microenvironment [43]. After a bone fracturing event, the bone healing process will be stimulated simultaneously and sequentially by a cascade of signaling pathways and cellular changes. This process consists of four consecutive steps, namely, inflammation, fibrocartilage callus formation, bony callus formation, and remodeling. Growth factors for each healing stage have temporal roles and should be delivered in sequence to achieve high efficiency [44,45].

The use of a single biomaterial via one delivery system would be insufficient for promoting such a complex physiological process. The fabrication of a suitable scaffold that can deliver multiple factors in a sustained and physiologically relevant temporal order is considered to have more capacity to mimick this complex physiological process. A wide range of delivery systems based on various biomaterials has been established for the multiple and sequential delivery of biomolecules [9]. Several scaffold manufacturing technologies that facilitate the release of multiple growth factors with special release profiles, such as polymer encapsulation, core-shell construction, and layer-by-layer fabrication, have been reported [46].

Polymer encapsulation: Polymer encapsulation is a common method to deliver multiple growth factors with specific profiles from a polymeric scaffold. Multiple and specific release profiles can be achieved by utilizing polymers with different swelling and degradation behavior to separately incorporate growth factors. The most important way is to utilize microsphere encapsulation to create controlled drug-releasing scaffolds with multiple material phases [47]. The drug loading microspheres are commonly fabricated using one polymer to implant into the scaffold material to form a second polymer. The protein burst release can be altered by adjusting the percentage of polymer content in the microspheres [47,48]. New mathematical models have been established to accurately estimate the release profiles of single molecules using polymer microspheres. In this model, PLGA microspheres have been designed and fabricated by these predictive methods to release a multitude of growth factors. Their presentation to the body will be delayed by regulating the properties of the polymer material such as its composition, polymer ratio, molecular weight, and the porosity of microsphere surface [49].

Core-shell construction: Utilizing core-shell technology is another approach to construct multiple and sequential delivery scaffolds. The shell layer is generally constructed by a polymer with a faster degradation rate, and the core layer is composed of one with a slower rate. The core-shell scaffold arrangement facilitates the sequential release by construction of the core-shell structure, which protects the drug released from the core. Different release profiles are generated depending on the location of the protein (core or shell) and the phase composition of the shell or core layer (such as percentage and crosslinking degree). The mechanism for release partially depends on the diffusion through both the core and shell materials [50]. Although the core–shell fiber design is effective in creating different release profiles, one of the possible limitations for multiple growth factor release is the change to the material matrix to modulate the release profile. This phenomenon leads to the sacrifice of several desirable material properties. Hence, to avoid this problem, the growth factors are encapsulated into polymer microparticles and then implanted into secondary scaffolding loaded with another growth factor. In this case, the microspheres are used as the "core" phase and incorporated into the "shell" scaffolding. This process is elegantly described by Kempen *et al*. in their work regarding the release of bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) [51–54].

Layer-by-layer (LbL) fabrication: The LbL is an advance technique that delivers multiple growth factors in a sustained and controlled manner. This technique relies on hardened layers of biomaterials or polymers applied directly on top of each other. Therefore, different kinds of growth factors are dispersed in diverse layers to control its release. This technique makes use of material characteristics to release multiple growth factors by transforming the curing process of each layer to regulate release rules [55]. To minimize burst release even further, the growth factor can be initially encapsulated into microspheres, and then these microspheres are included in subsequent layers [55,56].

New methods to produce scaffolds using LbL have shown this method can create layers with increased levels of co-localized growth factors [57]. The LbL method allows the production of considerably thin layers to form an entire scaffold.

The gradients of growth factors are significant in LbL applications for vascularized bone. One challenge of the LbL method is to maintain the balance between the thickness of the different layers and the spatial separation of growth factors in question. Several reports have described that the molecular release is not spatially or effectively separated throughout the scaffold. For continuous release profiles, a barrier layer is necessary to prevent interlayer diffusion [58,59].

Scaffolds embedded with cells

Cell-based therapies currently depend on cell seeding and culturing with pre-fabricated scaffolds, but this method is considered time consuming, inefficient, and operator dependent. Furthermore, seeding the cells on these materials rarely recreates the cellular organization and does not address the vascularization construction issues. In this case, spatial distribution of manually seeded cells is random. To address these issues, cells are incorporated into the scaffold to create living cell/biomaterial/biomolecule constructs during the AM process. This technology is known as bioprinting or biofabrication and has been steadily increasing in popularity over the last decade [12,60–62].

A robust deposition is vital to the success of bioprinting, but difficulty arises because of the requirement to dispense cell-blending media. This drawback reduces the range of feasible techniques due to the requirement to work in an aqueous environment and most notably in the strict requirement to work within a tight temperature range (room temperature to 38 °C). Consequently, most major studies on bioprinting used inkjet-based cell printing, laser-assisted cell printing, and microextrusion [60].

Inkjet-based bioprinting: Inkjet-based cell printing was the first bioprinting technology to be reported. Inkjet bioprinting physically manipulates a bioink solution (a hydrogel pre-polymer solution with encapsulated cells) to generate droplets. This process utilizes gravity, atmospheric pressure, and fluid mechanics of the bioink solution to spray droplets onto a receiving substrate [63].

Inkjet bioprinting presents numerous advantages, the most important of which is the relatively high cell viability (usually from 80% to 90%), as determined by many experimental results [64–66]. Furthermore, this process can generate microscale constructions with deposited cells without sacrificing cellular viability or inducing damage to the phenotype or genotype.

This approach offers the possibility of multiple print heads being used to simultaneously print multiple cell types with biomolecules embedded within these biomaterials. Therefore, fabrication of complex multicellular constructs could be achieved by inkjet-based bioprinting. However, the cutting edge bioink printing is predominantly based on micro-electromechanical system (MEMS) devices. The MEMS-based printer heads are limited by their inability to dispense high-viscosity bioinks, and they do not work well with bioinks with high cell density, which will increase the viscosity of bioinks, resulting in the clogging of the head. Furthermore, cells gradually settle in the cartridge over the entire printing process, increasing the likelihood of a clogged system.

To date, the use of inkjet printing remains a relatively underexplored field. Cui *et al*. superbly describes a series

of BTE scaffolds obtained through inkjet printing. Human mesenchymal stem cells (HMSC), photopolymerizable PEG-based polymer, and HA nanoparticles were simultaneously bioprinted. The nanoparticles closely mimicked the native tissue microenvironment and stimulated the differentiation of stems cells toward osteogenic lineage [67]. They also developed acrylated peptides and co-printed with acrylated poly (ethylene glycol) (PEG) hydrogel with simultaneous photopolymerization to minimize the nozzle clogging [68]. Subsequent work from the same group explored inkjet bioprinted-HMSCs in simultaneously photo-crosslinked hydrogel scaffolds. These scaffolds evidently improved the mechanical properties and increased chondrogenic and osteogenic differentiation. These results suggest the potential of this technique for fabricating BTE scaffolds [69].

Extrusion-based bioprinting (EBB): EBB bioprinting is considered a modification of inkjet printing. Given that viscous materials are often not suitable for printing with inkjet printers, extrusion printing uses either an air-forced pump or a mechanical screw plunger to dispense more viscous bioinks. Almost all kinds of hydrogel solutions with diverse viscosity and aggregates with high cell density can be printed by extrusion bioprinters [70]. Although EBB is the most convenient technique for fabricating 3D porous cellular structures rapidly, this technique has several limitations, such as low resolution, cell deformation induced by shear-stress, and limited material selection [71,72].

EBB is currently utilized in diverse fields. However, this technique is still insufficient, especially for cell-embedding scaffolds. One study [73] described several constructs consisting of alginate supplemented with mesenchymal stem cells (MSCs). The authors printed CaP particles by EEB bioprinting, with plasmid DNA encoding BMP-2 in the constructs. *In vitro* studies have shown that cells were efficiently transfected and differentiated toward the osteogenic lineage. However, *in vivo* experiments showed no bone formation after six weeks of subcutaneous implantation in nude mice. This example, although promising, demonstrates that EBB requires further extensive optimization before this technique can be widely applied in a clinical setting [74].

Laser BioPrinting (LaBP): LaBP depends on laser-induced forward-transfer [75] and consists of three sections, namely, a pulsed laser source, a ribbon coated with liquid biological materials deposited on a metal film, and a receiving substrate [76]. The lasers irradiate the ribbon to evaporate the liquid materials which arrive at the receiving substrate in droplet form. The receiving substrate includes a biopolymer and culture medium to maintain cell adhesion and growth after transferring the cells from the ribbon. This cell-by-cell deposition method theoretically enables the precise micro of the cells and potentially improves cellular interactions [61]. LaBP has already been applied in the printing of nano-HA (nHA) and human osteoprogenitors (HOPs) in two and three dimensions. The results showed no alteration in the viability, proliferation, and phenotype of HOPs after 15 days [77]. Another study utilized laser-induced jet dynamics to determine the optimum conditions from which jets arise with minimum kinetic energies. Using this method, MSCs with high viability can be harnessed to print [78].

Bioprinting methods are generally limited in their ability to generate large biological constructs with sufficient structural integrity for surgical implantation [62]. To our knowledge, the *in vivo* studies associated with bioprinted scaffolds applied to BTE are still lacking. However, this area is gradually becoming more explored as more groups become interested in the potential of bioprinting. All reported studies show that the osteoinductive and osteoconductive properties of the scaffold improved [79,80].

Scaffolds designed with pre-vascularized structures

Although several advances have been achieved toward cell-based approaches and growth factor delivery systems, these scaffolds often lack control over the arrangement of the engineered vascular networks in 3D scaffolds. These techniques usually lead to randomly organized network structures, resulting in inefficient distribution of oxygen, nutrients, and waste products in the 3D tissue constructs [81]. Vascular networks are highly organized and are hierarchically structured in tissues. Hence, coupling micro environmental cues with microfabrication methods is a promising alternative to engineer pre-vascularized structures inside these scaffolds.

Thus, fabrication approaches to engineer pre-vascularized scaffolds with precise and pre-defined microvascular channels have been developed. Soft lithography and rapid-prototyping are currently the most explored techniques to manufacture interconnected and spatially organized pre-vascular networks. Computer-aided technology is also used to predict oxygen consumption and pressure distribution in a certain scaffold, which will satisfy the requirements of different scaffolds and provide guidance on the overall fabrication process [82].

Soft lithography: Soft lithography is a collection of techniques based on printing, molding, and embossing with an elastomeric stamp [83]. Soft lithography includes numerous patterning techniques, such as micro contact printing, replica molding, and microtransfer molding. All these techniques use polymeric materials that are referred to as soft matter. Nikkhah *et al*. [84] fabricated micropatterned gelatin methacrylate hydrogels with varying geometrical features (50–150 μm height) by soft lithography methods. They primarily investigated the 3D endothelial cord formation within the scaffold. This work demonstrated that human umbilical vein endothelial cells could align and arrange within the micropatterned

constructs and assemble to form cord-like structures. Similar studies by Raghavan *et al*. [85] present scaffolds using microfabricated poly (dimethylsiloxane) templates to spatially arrange endothelial cells (ECs) within collagen gels to promote endothelial cord formation. Another study also utilized the lithographic technique to engineer living microvascular networks in 3D tissue scaffolds and demonstrated their biofunctionality *in vitro*. Further reports described the spontaneous formation of tubular constructs and angiogenic sprouting when perivascular cells and smooth muscle cells were encapsulated in the matrix [86]. Additionally, soft lithography has been shown to work well when combined with microfabrication to create microchannels in a porous silk-based matrix [87]. All these results show that ECs reached good confluence and proliferated well within the highly porous areas.

Rapid prototyping: Rapid prototyping can be used to construct bionic BTE scaffolds directly as previously described. This technique also represents one of the most effective methods for fabricating 3D sacrificial templates. This approach involves printing a primary well-designed 3D template structure through rapid prototyping, and this 3D template functions as a mold for a secondary biomaterial with or without cells. After the secondary matrix is cross linked, the primary sacrificial template is then removed by physical, chemical, or thermal methods. This process generates a 3D scaffold with highly organized interconnected microchannels.

Miller *et al*. first reported this approach in 2012 [88]. They printed rigid 3D tubular networks using carbohydrate glass as a template which was subsequently removed to generate cylindrical networks, in which ECs and blood were perfused under high-pressure pulsatile flow. Meanwhile, Bertassoni *et al*. printed agarose templates to form hollow cylindrical channels in pre-osteoblast cell-laden tissue constructs [89]. The results showed significant improvement in cell viability and higher alkaline phosphatase expression by the pre-osteoblast cells. More importantly, by seeding ECs, these embedded channels acted as a provision for organized vasculature by facilitating the sprouting of cells lining the lumen in the surrounding matrix. A recent report introduced laser-sintered carbohydrate materials as templates for vascular networks because of the rigid, self-supporting, water-soluble, and biocompatible characteristics of these materials. Hence, 3D vessel networks can be created by encasing the templates in diverse hard and soft ECM materials and then dissolving away the templates [90].

However, 3D sacrificial template approaches are only adapted to construct simple interconnected microchannel architectures consisting of homogeneous cell-laden matrices. Natural tissues have a more complex structure and comprised multiple cell types. Kolesky [91] reported an improved method for fabricating engineered tissue scaffolds with vasculature, multiple types of cells, and ECM. In this work, the authors developed an aqueous fugitive ink composed of pluronic F127 which can be easily printed and removed under mild conditions.

Although these pre-vascularized methods cannot mimic the complex structure and dynamic steps involved in the formation of the vascular system, the oxygen and nutrient diffusion and waste removal properties of the scaffolds were greatly improved in the cell-laden tissue constructs through this fabrication of the vascular network.

In conclusion, we recommend that different tissue fabrication technologies should be integrated to achieve fully pre-vascularized, biomimetic, and load-bearing BTE scaffolds in the future.

Cells in BTE

Seed cells are essential elements for tissue engineering applications, such as bone regeneration. BTE has a number of potential cells, such as osteoblasts, preosteoblasts, and stem cells. The stem cells in bone regeneration are divided into adult stem cells, embryonic stem cells (ESCs), extraembryonic stem cells, and induced pluripotent stem cells (iPSCs) (Table 2). Bone marrow-derived MSCs (BMSCs) have been widely applied in BTE, while other alternatives from adult tissues, such as adipose tissue (AT), muscle, synovium, umbilical cord blood (UCB), Wharton's jelly, amniotic fluid, urine, dental pulp, and periosteal tissue, have been explored [92–97]. In this section, we will describe the tissue origins of bone progenitors, osteogenic differentiation, and the interconnection biology of the cells and the scaffolds during BTE (Fig. 2).

Adult stem cell sources

Adult stem cells have shown considerable ability for bone regeneration because of their potential multipotency, osteogenic capacity, and less ethical constraints. A better understanding of these cells will facilitate the selection of superior seed cells. However, compared with ESCs, stem cells possess several disadvantages, such as limited self-renewal capacities and lower proliferation and differentiation rates [98].

Bone marrow-derived mesenchymal stem cells (BMSCs)

BMSCs can differentiate into multiple mesenchymal lineages, such as chondrocytes, osteoblasts, osteocytes, adipocytes, and several other cell types. The differentiation of multipotential BMSCs to osteoblasts is fundamental for engineering application of bone tissue. The bone marrow is generally obtained from the marrow cavity during orthopedic surgical procedures or bone marrow aspiration.

Table 2 Characteristics of the different stem cells

Cell sources	Origin	Differentiation medium	Main markers
Adult stem cells			
BMSCs	Bone marrow	10–100 nmol/L DEX, 0.1–0. 5 mmol/L ACS, 10 mmol/L β-GP	CD105, CD106, CD73, CD90, CD45$^-$, CD34$^-$
ADSCs	Adipose tissue	10–100 nmol/L DEX, 10 mmol/L β-GP, 50 μmol/L ASP	CD29, CD34, CD73, CD90, CD105, CD106$^-$
MDSCs	Skeletal muscle	0. 5 mmol/L ACS, 5 mmol/L β-GP, 10 nmol/L DEX	CD44, CD73, CD90, CD105, Sca-1
SDSCs	Synovium	10 nmol/L DEX, 20 mmol/L β-GP, 50 μmol/L ASP	CD44, CD73, CD105, CD166, CD14$^-$
DPSCs	Pulps	100 nmol/L DEX, 0.5 mmol/L ACS, 10 mmol/L β-GP	CD44, CD90, CD34, CD166
USCs	Urine	100 nmol/L DEX, 10 mmol/L β-GP, 50 μmol/L ASP	CD44, CD73, CD90, CD105, CD133, CD45$^-$, HLA-DR$^-$
ESCs	Embryo	100 nmol/L DEX, 10 mmol/L β-GP, 0.5 mmol/L ACS	CD9, Oct-4, SOX2, SSEA3/4 and NANOG, TRA-1-60/81, SSEA1$^-$
Extra-embryonic stem cells			
UCBSCs	Umbilical cord blood	100 nmol/LDEX, 10 mmol/L β-GP, 0.5 mmol/L ACS	CD29, CD44, CD54, CD73, CD90, CD105, CD49d
Wharton's Jelly SCs	Umbilical cord	2 mmol/L L-glutamine, 100 nmol/L DEX, 10 mmol/L β-GP, 0.2 mmol/L ACS	CD34, CD45, CD44, CD73, CD90, CD105, HLA-DR$^-$, CD18$^-$
AFSCs	Amniotic fluid	100 nmol/L DEX, 10 mmol/L β-GP, 0.5 mmol/L ACS	CD31, CD44 CD45, CD90
PDSCs	Placenta	100 nmol/L DEX, 10 mmol/L β-GP, 20 μmol/L ASP	CD29, CD73, CD166, CD34$^-$, CD45$^-$, SSEA-1/4, Oct-4, Sox2
AMSCs	Amnion	100 nmol/L DEX, 10 mmol/L β-GP, 100 μmmol/LASC	Oct-4, Nanog, CD34, CD90, CD105
IPSCs	Somatic cells	100 nmol/L DEX, 10 mmol/L β-GP, 100 μmol/L ASP	CD29, CD34, CD44, CD73, CD90, CD105, Sox2, TRA-1-81, Oct3/4

Dex, dexamethasone; β-GP, β-glycerophosphate; ASC, ascorbic acid; ASP, ascorbate 2-phosphate.

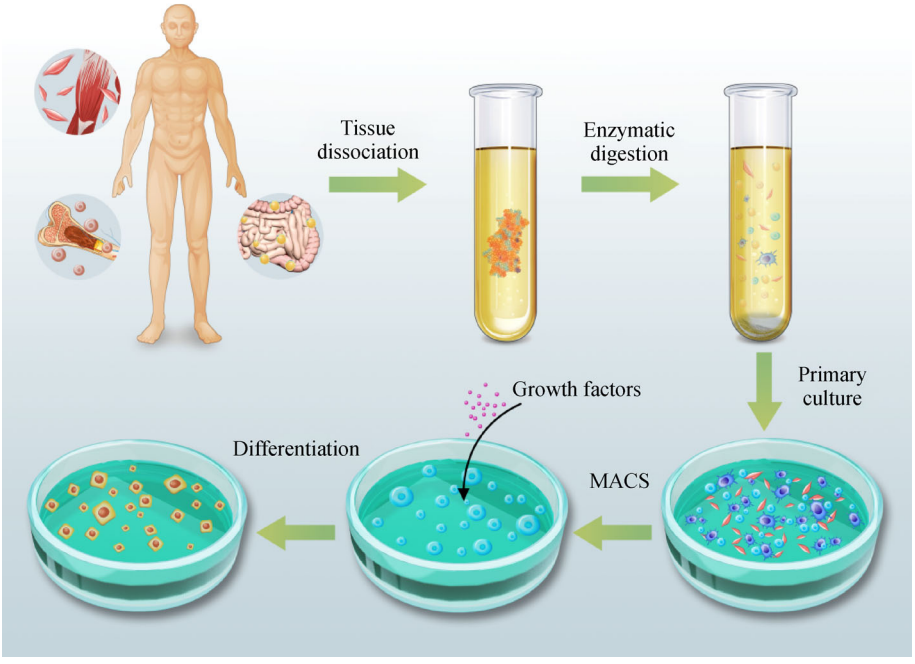

Fig. 2 Extraction processes and osteogenic differentiation of stem cells in the BTE. The tissues are first dissociated from the patients and digested with enzymes. The primary cells are cultured and followed by magnetic activated cell sorting (MACS). Those stem cells are differentiated into osteocytes with the help of growth factors.

Then, the stem cells are separated from the bone marrow samples and immediately resuspended in a MSC culture medium. The commonly used complete medium usually contains Dulbecco's modified Eagle's medium (DMEM) of low glucose, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured in a humidified incubator of 37 °C and 5% CO_2. BMSCs were purified on the basis of the ability to adhere on plastic substrate and high proliferation. Meanwhile, non-adherent cells are removed by changing the medium. When the BMSCs reached about 90% confluence, the cells are subcultured and expanded for further experiments. The BMSCs were identified by positive expression of valid molecular markers, such as CD105 (endoglin), CD106, CD73, and CD90, and negative expression of CD45, CD34, and HLA-DR molecular phenotype [99]. Although BMSCs can self-renew, the extremely low cell proportion in bone marrow cells and reduced differentiation capacities with aging are great challenges for tissue engineering [100,101]. To induce osteoblastic differentiation, the subcultured cells are usually cultivated in an MSC culture medium supplemented with 10–100 nmol/L dexamethasone, 0.1–0.5 mmol/L L-ascorbic acid, and 10 mmol/L β-glycerophosphate for two weeks. Following osteogenic induction, BMSCs showed osteoblast-like appearance and positive expression of alkaline phosphatase.

Adipose-derived stem cells (ADSCs)

ADSCs can differentiate into chondrogenic, adipogenic, and myogenic lineages, as well as exhibit osteogenic characteristics similar to BMSCs. However, compared with the bone marrow, the AT can be easily obtained using minimally invasive surgical procedures. In addition, the adipose cell yields are significantly high [102]. For instance, a liposuction procedure that is frequently used to acquire fat tissue is less invasive than bone marrow aspiration [103]. The routine to separate ADSCs from fat tissue relies on the digestion with collagenase or trypsin and the following centrifugation [104]. Despite the relatively higher stem cell density in ATs, the heterogeneous cell population of the stromal vascular fraction confines the effective isolation of ADSC at source [105]. In addition, the contamination of the cells may reduce proliferation and differentiated proficiency. These characteristics limit the application of ADSCs for tissue regeneration. Moreover, ADSCs could be roughly discriminated from other mixed cells through morphology and phenotype. ADSCs have fibroblast-like morphology and show positive expression of CD29, CD49d, CD73, CD90, and CD105 but negative expression of CD106, that is extensively expressed in BMSCs [106]. Isolated ADSCs were generally expanded in primary cultured conditions and then replaced by osteogenic differentiation medium, in which the culture medium is usually supplemented with 50 μmol/L ascorbate 2-phosphates, 10 mmol/L β-glycerol-phosphate, and 10 – 100 nmol/L dexamethasones.

Muscle and synovium-derived stem cells

Muscle-derived stem cells (MDSCs) have been found in skeletal muscles and are classified as conventional stem cells because of their self-renewal and differentiation as mesodermal progenitors. The modified preplate technique is the most common isolation method for a population of MDSCs. The preplating technique is used to separate MDSCs on the basis of their slowly adhering rates to tissue culture plastic and harvest MDSCs following 5 days of adherence [107]. Stem cells are initially released by enzymatic digestion and are purified via further density gradient centrifugation. They show phenotypes similar to those of BMSCs, including CD73, CD90, CD105, CD44, and Sca-1, but lack the CD45 marker [108]. The osteogenic medium contains various osteogenic supplements, mainly composed of 0.5 mmol/L ascorbic acid, 5 mmol/L β-glycerophosphate, and 10 nmol/L dexamethasone.

Synovium-derived stem cells (SDSCs) display a higher proliferative rate and colony-forming efficiency compared with BMSCs [109]. However, Fan *et al*. reported that the osteogenic differentiation capacity of SDSCs was lower than that of BMSCs [110]. The synovium was usually obtained from the articular cavity and digested with collagenase and dispase. After digestion and subsequent expansion, SDSCs were cultured under osteogenic conditions of 10 nmol/L dexamethasone, 20 mmol/L β-glycerol phosphate, and 50 μmol/L ascorbate-2-phosphate for an additional three weeks. As fibroblasts and macrophages are frequent contaminants in primary cultures, the isolated SDSC yields a heterogeneous population. Hence, the surface marker of cultured stem cells is characterized in the positive expression of CD44, CD73, CD105, and CD166 but negative expression of CD14 and CD45, which could eliminate the fibroblasts and stromal lineages [108].

Dental pulp stem cells (DPSCs)

DPSCs have the advantages of efficient self-renewal and specific differentiation ability. These cells could differentiate into chondrocyte cells, osteoblast cells, ECs, and adipocytes [95,111]. The pulps were acquired from normal immature molars and then were cut into tiny pieces for culture. DPSCs can be isolated from cultured pulp cells by detecting surface stem cell markers, such as the positive expression of CD44, CD90, CD34, and CD166 [95]. DPSCs can differentiate into osteoblast precursors and then into osteoblasts in a complete medium supplemented with 0.5 mmol/L ascorbic acid, 100 nmol/L dexamethasone, and 10 mmol/L β-glycerolphosphate for one week.

Urine-derived stem cells (USCs)

Urine is an effective source of stem cells called USCs because these cells are easily obtained in large volumes. Recent studies have demonstrated that these cells possess features similar to those of ADSCs, which express typical surface markers of MSCs, but with higher proliferation capacity than ADSCs [112]. Sterile urine samples were harvested and centrifuged, and the obtained cell pellets were resuspended with MCS culture medium. USCs have exhibited the positive expression of CD34, CD44, CD45, CD73, CD90, and CD133 but negative expression of CD45 and HLA-DR makers [96]. To induce osteogenic differentiation, USCs were seeded with culture medium containing of 50 μmol/L ascorbate 2-phosphate, 10 mmol/L β-glycerol phosphate, and 10 nmol/L dexamethasone for 3 weeks.

ESCs

The discovery and isolation of ESCs propel the stem cells into new directions because of their infinite self-renewal activities and differentiation into other cell lineages from three germ layers for tissue engineering. These stem cells originated from the inner cell mass of the embryo during the blastocyst stage, and the osteogenic lineage cells with osteogenic capacity are derived from the somatic mesoderm of mesodermal progenitor cells [113,114]. To isolate these cells from the fertilized embryoid bodies, the embryo should be cultured until the blastocyst stage so that cell masses can be completely isolated. Typically, the inner cell mass of the blastocyst is mechanically isolated using a specially plated needle, and the trophectoderm cells are subsequently removed to transfer the inner cell mass onto fresh feeder plates [115]. Contrary to adult stem cells, ESCs are grown on tissue culture plastic containing embryonic fibroblast feeder layers and the cultured medium consisting of DMEM, 20% serum replacement, 3.5 mmol/L L-glutamine, 100 μmol/L β-mercaptoethanol, and 10 ng/mL bFGF for one day. The ESCs are then induced to the osteoblasts of mesenchymal lineage in osteogenic differentiation medium for 7 days. ESCs have expressed specific markers of CD9, Oct-4, SOX2, SSEA3/4, and NANOG, which express a more complex phenotype than BMSCs. However, ESCs have shown prominent tumorigenic properties which lead to potentially infinite proliferation and teratoma production. Therefore, precisely controlled ESC differentiation along the osteogenic lineage and optimal osteogenic conditions *in vitro* are considerably essential and urgent.

Extra-embryonic stem cells

Similar to ESCs, cells from extra-embryonic sources can regenerate somatic cells for tissue engineering. These cells are usually isolated from tissues discarded after birth, such as Wharton's Jelly stem cells, UCB stem cells, amniotic fluid stem cells (AFSMs), and amnion-derived stem cells (AMSCs). These cells display a more primitive state and multi-lineage differentiation capability.

UCB stem cells

UCB, as well as bone marrow containing hematopoietic stem cells and MSCs, can differentiate into different cell types which show intermediate characteristics between embryonic and adult stem cells [116]. Samples of umbilical blood are harvested from the umbilical cord after the delivery and cord clamping but before placenta expulsion. UCB stem cells are separated into the hystopaque, and mononuclear cells are isolated by ficoll density centrifugation and suspended in culture medium. These cells are positive for various surface markers, such as CD29, CD44, CD54, CD73, CD90, and CD105, which is homologous to other MSCs. However, the expression of CD49d has been observed in UCB stem cells but not in BMSCs. For osteogenic differentiation, the cultured cells were transferred to the complete medium supplemented with 10 mmol/L β-glycerophophate, 0.1 mmol/L dexamethasone, and 50 mmol/L ascorbate for 4 weeks.

Wharton's jelly stem cells

Stem cells derived from the umbilical cord without inner membrane named Wharton's jelly possess desirable characteristics and represent a prospective cell source for tissue regeneration. The advantages of Wharton's jelly stem cells include rapidly available samples, noninvasive procedures, ethically non-controversial, and no tumorigenic induction [117]. These cells are obtained from the umbilical cord after full-term births, and to isolate the stem cells, the tissue is aseptically cut into small pieces and plated in plastic with complete medium of suspension culturing for 7 days. For cell differentiation, Wharton's jelly stem cells are incubated in osteogenic medium supplemented with 2 mmol/L L-glutamine, 0.1 μmol/L dexamethasone, 10 mmol/L β glycerol- phosphate, and 0.2 mmol/L ascorbic acid. These stem cells were determined to express surface markers of CD34, CD45, CD44, CD73, CD90, and CD105 but not HLA-DR and CD18 expression. Thus, the osteogenic potential of these cells is demonstrated [117,118].

AFSCs

Amniotic fluid is frequently used because it contains multiple cell types derived from the developing fetus. The majority of cells within this heterogeneous population, such as AFSCs, are pluripotent cells that show wide

differentiated activity to diverse cell lineages [119]. AFSCs can be easily harvested from a small volume of second trimester amniotic fluid collected during routine amniocenteses for prenatal diagnosis [120,121]. AFSCs are isolated from the amniotic fluid through centrifugation and medium suspension, and the detached cells are subsequently collected and transferred to a new plate for primary culturing. AFSCs can also be isolated using a selection based on expressing the surface antigen c-Kit (CD117) [120]. AFSCs show positivity for specific markers of two MSC surface markers of CD44 and CD90, as well as two hematopoietic stem cell surface markers of CD 31 and CD 45 [122]. AFSCs are maintained in osteogenic induction medium consisting of 100 nmol/L dexamethasone, 10 mmol/L β-glycerol phosphate, and 0.5 mmol/L ascorbate for 3 weeks.

Placenta-derived stem cells (PDSCs)

Stem cells isolated from the placenta have been extensively used because of their wide availability, the absence of ethical disputes, low immunogenicity, low oncogenicity, and multipotent differentiation under controlled conditions [123,124]. The placenta cells synthesize diverse hormones, enzymes, and growth factors. Thus, PDSCs can secrete biologically active substances to promote bone repair [125]. The tissues are typically obtained from the maternal placenta under sterile conditions, minced into fragments, and then digested with collagenase II solution. The mixture fluid is centrifuged, and the released cells are resuspended with the culture medium. After 80% confluence, the stem cell culture medium is replaced with osteogenic differentiation medium for 2 weeks. Their surface markers are similar to those of BMSCs, with the exception of the positive expression of SSEA-1/4 and Oct-4, which are mainly expressed in ESCs [126].

AMSCs

AMSCs are considered as alternative resources in BTE. The noninvasive approach, fewer ethical issues, and the fast proliferation following greater differentiation potential could make AMSCs available for bone regeneration [127]. AMSCs can be harvested from amniotic membranes which are abundant tissues during operative deliveries. The ADSCs are minced into 2.5 cm pieces and isolated by digesting with collagenase I solution, followed by seeding in plastic dishes with complete medium. To promote osteogenic differentiation, the AMSCs are cultured in osteogenic medium, consisting of 10 mmol/L β-glycerophosphate, 100 nmol/L ascorbic acid, and 100 nmol/L dexamethasone. The expressions of specific markers in AMSCs contain Oct-4, Nanog, CD34, CD90 and CD105 [128].

IPSCs

IPSCs are derived from somatic cells that can be genetically reprogrammed into an embryonic stem cell-like state. As one form of embryonic-like stem cells, the iPSCs have drawn increasing attention because of their pluripotency and self-renewal capacity. Most importantly, iPSCs avoid causing the ethical constraint, security issues, and shortage of sources that are typical of ESCs [97]. IPSCs are generated by transducing the murine cells with four genes, namely, Oct4, Sox2, Klf4, and c-Myc, in the first instance. To generate human iPSCs, four transcription factors of Oct4, Sox2, Nanog, and Lin28 are transfected into human somatic cells [129–131]. The advances and valid characteristics of iPSCs have shown great potential for tissue engineering. The somatic cells, such as embryonic fibroblasts, are derived and then epigenetically reprogrammed into iPSCs through a polycystronic lentiviral vector. The iPSCs are produced and maintained in primate embryonic stem cell medium supplemented with serum replacement, L-glutamine, penicillin/streptomycin, β-mercaptoethanol, and nonessential amino acids. For osteogenic differentiation, human iPSCs are cultured in the osteogenic medium supplemented with 100 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, and 100 μmol/L ascorbic acid 2-phosphates for 3 weeks. The iPSCs exhibit high levels of MSC surface markers of CD29, CD34, CD44, CD73, CD90, and CD105, as well as the pluripotency markers of Sox2, TRA-1-81, and Oct3/4, consistent with surface marker expression to ESCs [132].

Coculturing cell system

The coculture technique is a cell cultivation method used to study the natural interactions with two or more different cells and aids in increasing the culturing success rate for certain cell populations. Compared with the conventional cell culture, coculture systems are considered as an effective and essential technique to enhance cell synthetic interactions and understanding as well as helping promote bone cell differentiation and bone regeneration.

Stem cells and EC coculture

Given the intimate connection between angiogenesis and osteogenesis, the communication between mesenchymal stem cells and ECs is a crucial cellular interaction for bone regeneration. The bone is a highly vascularized tissue, and an adequate blood supply is an important component to provide nutrition, oxygen, cytokines, and growth factors for bone repair in tissue engineering [133]. Osteogenesis is an intricate process affected by physiological conditions, cell interactions, matrix formation, and vascularization levels. Previous studies demonstrated that paracrine

growth factors, such as VEGF and fibroblast growth factor (FGF) are required to induce angiogenesis and mediate in the interactions for osteogenesis [134]. ECs play an important role in controlling the physiological and pathological procedure of permeability, endocrine, angiogenesis, and inflammation. Therefore, the coculture of vascular cells could provide multiple advantages compared with osteogenic cells alone in bone regeneration. ECs are usually derived from microvasculature, umbilical veins, large blood vessels, or kidney EC for generation of vascular structures [135,136]. The stem cells and ECs are isolated, primarily monocultured, and then combined and seeded together onto culture plastic in mixed culture and osteogenic medium. Xue *et al*. reported that coculture led to the upregulation of angiogenesis genes, such as von Willebrand factor (vWF), cell adhesion molecule-1 (CAM-1), cadherin 5 (CDH5) and CD34, but also upregulated the markers of osteogenesis, such as alkaline phosphatase (ALP) and bone morphogenetic protein (BMP) [137]. These signal molecules may play an important role in cell–cell adhesion and cell-ECM interaction. Nesti *et al*. demonstrated that bone matrix is rich in reservoirs of transforming growth factor-β (TGF-β), and cell–matrix interactions and TGF-β signal pathways participate in EC-induced gene regulation of MSCs [138].

Osteoblasts and EC coculture

Osteoblasts can secrete collagen matrices and proteins, such as osteocalcin and osteopontin, to participate in the mineralization during bone regeneration. Primary ECs and osteoblastic cells are isolated directly from tissues and cocultured in 3D biomaterial scaffolds. During the cell–cell interactions, VEGF receptors are upregulated in ECs, whereas an upregulation of ALP activity was observed in osteoblasts [139]. The sonic hedgehog pathway is regarded as one of the main signaling pathways that manage the angiogenesis and osteogenesis in the coculture system [141]. The coculture of ECs and osteoblast cells resulted in the network formation of capillary-like structures through the biomaterial scaffold, which may rely on the pro-angiogenic signaling of secreting VEGF and gap junction protein connexin-43 [140]. The capillary structures could provide a microenvironment of adequate nutrients and signaling molecules to influence osteoblast growth, proliferation, and matrix production.

BMSCs and periosteal-derived stem cells (PCs)

BMSCs are significant cellular sources for bone regeneration because of their osteogenic differentiation activity. However, BMSCs are limited by their small cell populations and limited proliferation and osteogenic potential [99,100]. PCs are usually isolated from the periosteum and commonly regarded as a core source for bone self-repair. These cells show strong osteogenic potential to form bone tissue. Fracture healing requires recruiting skeletal stem cells to permit the formation of osteochondral tissue which are assembled from the periosteum and bone marrow during bone repair [142]. Chen *et al*. observed that co-culturing of these two cell types show synergistic effects and accelerate bone regeneration through cellular responses [143]. Co-culturing BMSCs and PCs significantly increased the expression of COL1A1, osteopontin, osteocalcin, and BMP-2 to synthesize ECM and accelerate osteoblastic mineralization [144]. The adhesions and interactions of cells in a coculture system may affect cell–cell signaling to promote osteogenic differentiation.

Application of stem cells for BTE

Cell-based approaches using BMSCs and biomaterials have achieved remarkable success at healing the large bone defects in experimental bone regeneration *in vivo*. For instance, Park *et al*. utilized PLGA microspheres and BMSCs combined with laser therapy and observed a clear enhancement of the ectopic bone in mice [145]. Additionally, the combination of ECs and BMSCs with deproteinized biological bone has enhanced the bone repair capacity and improved early vascularization of segmental bone defects [146]. Recent clinical trials of long bone defects have reported the use of BMSCs, but the results are not entirely available and they reflect the obstacles to clinical applications of bone repair [147]. Similar to the BMSCs, multiple recent studies applied ADSCs on BTE both in animal models and in humans. However, animal studies of ADSCs are advantageous over the BMSCs, such as greater proliferation and reduced senescence *in vitro* [148,149]. Sandor *et al*. implanted ADSCs on a β - tricalcium phosphate (β-TCP) scaffold with the addition of BMP-2 to reconstruct the maxillofacial defect in 13 patients. The results showed 10 cases with successful bone regeneration [150]. To date, the main combination of other adult stem cells and biomaterial scaffolds has shown exceptional advancement in critical-sized defects of experimental animals or *in vitro* [110–113,117,124,126, 127]. Recent bone regeneration studies have shown the potential application of ESCs and iPSCs combined with 3D scaffolds for BTE because of the successful development in osteogenesis studies *in vitro*. Kuhn *et al*. reported that hESCs seeded onto HA scaffolds induced the formation of vascularized bone in calvarial defects of immunodeficient mice [151]. Effective bone formation was observed by Levi *et al*. [152] when they transferred iPSCs onto an HA and BMP-2 releasing scaffold for *in vivo* assessment.

Cell therapy has served as an alternative for bone regeneration by the injection of a number of bone

progenitor cells into the impaired bone defect in combination with a biomaterial matrix. BMSCs are the most frequently used cells for inducing bone repair by cell therapy. These cells have already been applied clinically for atrophic nonunion fractures in patients [153]. Other stem cells, such as DPSCs, are feasible cell-based therapies for bone defect of animal models [154]. However, at present, only autologous MSCs are clinically used for cell therapy in bone regeneration.

Bioactive factors

Bioactive factors are generally osteoinductive and angiogenic, and they form the integral components of traditional BTE. These factors stimulate one or several processes in bone formation and have been extensively studied in various clinical fields, such as fracture repair, spinal fusions, cranial defects, and dental procedures [155]. Through the connection between bioactive factors and its cell receptors, these factors provide a guiding role for cell differentiation or tissue regeneration via cell signaling pathways. According to their characteristics, these small compounds can be divided into four groups, namely, growth factors, peptides, chemical molecules, and other small molecules (Fig. 3).

Growth factors

TGF-β

The TGF-β (TGF-β1 to TGF-β5) superfamily have five isoforms, namely, BMPs, growth differentiation factors (GDFs), activins, inhibins, and Mullerian substances [156]. The effect of TGF-β on cell growth, differentiation, and ECM synthesis is well established. However, for bone repair and regeneration, TGF-β is remarkably underexplored, with studies focusing on experimental animal models. For example, Joyce *et al*. [157] used a subperiosteal injection in an animal model and showed that the injection of TGF-β1 can stimulate periosteum cells to endochondral ossification. Lind *et al*. [158] performed continuous TGF-β infusions on unilateral fractures of the tibia in rabbit models. The results also demonstrated a significant increase in the formation of bone calluses. However, not all related studies have obtained the same conclusions. Critchlow *et al*. [159] evaluated the effect of TGF-β2 on the bone repair of rabbit tibial fractures under different surgical treatments. Both groups accepted the injection of TGF-β2 with higher (600 ng) or lower (60 ng) dose. Consequently, after treatment with the high dose, abundant calluses without less bone calcium content appeared in stable group, but the test subjects administered

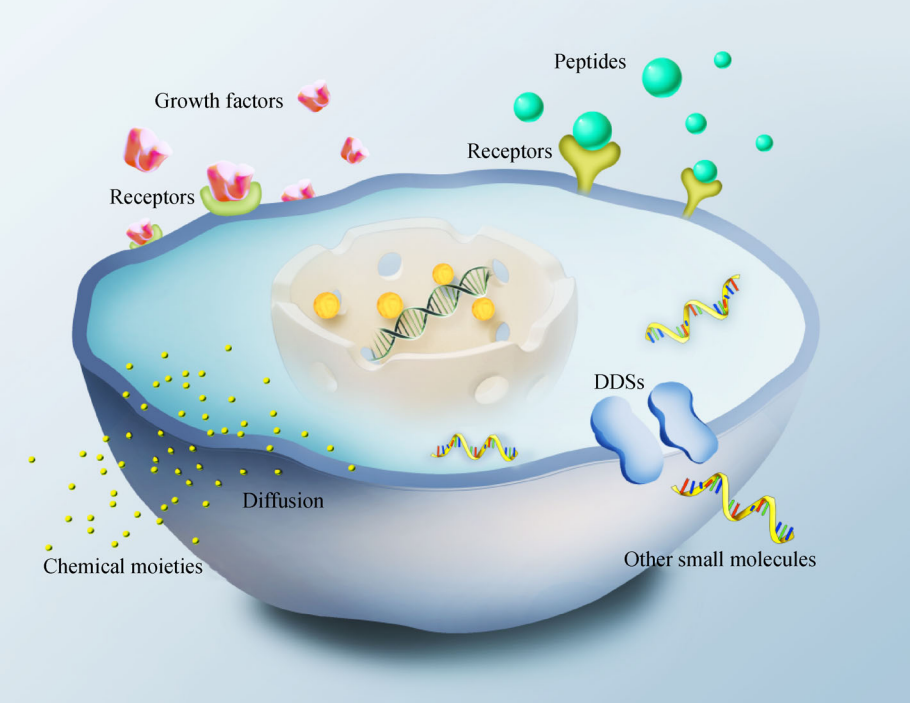

Fig. 3 Classifications and action patterns of bioactive factors in BTE. Bioactive factors can be divided into four parts, namely, growth factors, peptides, chemical molecules, and other small molecules, according to their characteristics. These factors present distinct actions on the cytoplasmic and nuclear components, such as receptors, diffusion, and drug delivery systems.

with the lower dose showed insignificant effects on fracture repair. Hence, given the wide variability of the isoforms of the TGF-β superfamily used in these studies any firm conclusions on the efficacy of TGF-β to initiate the bone regeneration process is difficult to draw. Furthermore, although TGF-β has been demonstrated to enhance cellular proliferation, its osteoinductive potential need to be further verified in a clinical setting.

BMPs, which are a member of TGF-β superfamily, play an important role in the cell, such as human embryonic development, muscle development, and bone and cartilage formation [160], with BMPs being successfully used for bone defect treatment in the clinic. BMPs activate intracellular signaling cascades by binding with their specific cell surface receptors (type I and II serine/threonine kinase receptors). The Smads signaling pathway is initiated by the BMPs-receptor compound and actively recruits and phosphorylates the transcription factors Smad1, Smad5, and Smad8. These phosphorylated proteins are subsequently combined with Smad4 to transduce the signaling to the cell nucleus, and this process leads to osteoblastic gene expression [161]. A large number of cellular proteins have been identified as specific negative regulators of the BMP signaling pathway. For example, the E3 ubiquitin ligase for Smurf1, BMP antagonists (noggin, gremlin, and chordin), and antagonistic Smads (Smad6 and Smad7) have been implicated as downregulators of this pathway [162]. However, the activators have not yet been reported. The members of the BMPs family, such as BMP-2, 6, 7, and 9, have been investigated for their osteogenic capacity *in vitro* and *in vivo* [163–165]. In addition, the application of novel biomaterials in conjunction with these small polypeptides improves the potential for bone repair. Takahashi *et al*. showed that biodegradable gel materials macerated with BMP-2 could renew skull bone defects in monkeys [162]. Moreover, Wang *et al*. delivered both BMP-2 and BMP-7 using an implanted collagen sponge in a mini-pig calvarial defect model. These results demonstrated that the bone formation rate of this combined group increased by 1.5-fold compared with the single factor group [166]. BMP-2 and BMP-7 have been approved for specific clinical application by FDA because of their clear clinical effect on bone formation. BMP-6, which is expressed in osteoblasts, has also been demonstrated to stimulate the differentiation of preosteoblasts to form a calcific bone matrix during the early stage of bone fractures.

However, these small peptides consistently have drawbacks and side effects, such as high clinical cost, uncertain stability or activity, undesirable immune response, and high dose requirement. Some limitations can be partially overcome using peptides with similar sequence to BMP. Short peptides possess smaller sizes and a considerably lower immune response rate [167]. Moreover, this partial sequence of BMP is more stable and easier to produce than full-length BMP. For instance, the BMP-2-derived peptide (P24) can enhance osteogenesis both *in vitro* and *in vivo*. Li *et al*. observed that P24 specifically promoted bone defect repair in cranial defects in rat models [168]. At present, BMPs are the most widely studied small peptides in bone regeneration treatment in humans and experimental animal models. However, their clinical efficacy and safety still requires large-scale studies to determine whether they can have long-term applications in this setting.

FGFs

According to the affinity for the glycosaminoglycan heparin-binding sites on cell receptors, the FGF family is divided into nine structure analog polypeptides. FGF plays an important role in angiogenesis and mesenchymal cell mitogenesis [169]. FGF performs a number of functions within the cells, including bone regeneration precursor cells, such as regulation, proliferation, migration, and differentiation. Among these cells, FGF-1 and FGF-2 can regulate several steps of angiogenesis and bone repair in the early stages of fracture. For instance, Kato *et al.* [170] estimated the efficacy of single local recombinant human FGF-2 (rhFGF-2) on tibial defects in rabbits. Furthermore, dose-dependent effect was observed on bone repair of bone volume and bone mineral content. Additionally, FGF-2 can also directly or indirectly induce angiogenesis through VEGF/VEGFR signaling pathways. Several FGF ligands, such as FGF-2, 9, and 18, as well as their specific receptors FGFR-1, 2, and 3, are expressed in bone regeneration [171] and fracture repair [172]. Recent studies showed that FGF-9 was necessary for the reconstruction of blood vessel networks during fracture healing [173]. To date, FGFs have demonstrated their effects on the establishment of new vascular systems and bone mineral deposition.

Insulin-like growth factors (IGFs)

IGFs are multifunctional cell proliferation regulators and play an important role in promoting cell differentiation and individual development. In this family, IGF-1 and IGF-2 participate in bone formation. The effect of IGF-1 on bone fracture healing had been verified in both rat model and clinical settings [174]. Thaller *et al*. continuously delivered single IGF-1 for calvarial defect repair in rat models [175]. The results indicated that the IGF-1 injection group showed more intramembranous bone formation compared with the saline placebo group. IGF-1 may improve bone fracture repair through intramembranous ossification. Although IGF-2 is known to be the most highly expressed growth factor in the bone tissue, further work should be performed to fully explore and understand its role in promoting fracture healing.

VEGF

Bone fracture repair requires accurate control over several complex processes involving several phases and collaboration of osteogenesis and angiogenesis factors around the bone marrow, calluses, and surrounding hematoma. The vascularization should ensure the formation of blood vessels to transport nutrients and growth factors to fracture site. One of the most important factors for neovascularization is VEGF, acting as the indispensable regulator to activate angiogenesis, osteoblast differentiation, osteoclasts recruitment, and bone resorption activity [176]. During the early phases of bone healing, the potential for VEGF-mediated hematoma formation during angiogenesis is high [177]. Inhibiting VEGF activity resulted in impaired bone repair of femoral fractures, whereas local VEGF application improved successful bone repair in a rat model [177]. Based on current studies, VEGF can influence bone repair by directly or indirectly affecting bone precursor cells. First, VEGF induces bone precursor cells migrating consistently along new vessels to the fracture callus, where they differentiate into osteoblasts. Then, VEGF stimulates ECs to produce osteogenic cytokines that can facilitate the progenitor cell differentiation into osteoblasts [178]. Furthermore, osteoblasts that can produce VEGF can precisely regulate their own proliferation and differentiation [179]. The combination of two or more growth factors has become extensively investigated because of the high efficiency and synergistic effect of these factors. For example, bone scaffolds coated with both VEGF and BMP-6 can lead to higher ALP activity, growth of new blood vessels, and obvious bone tissue mineralization when compared with the independent application of the growth factors. Cui *et al*. also demonstrated that single VEGF can accelerate angiogenesis, while combined VEGF and BMP-6 can additively enhance both angiogenesis and osteogenesis *in vivo* [180].

Peptides

Osteogenic growth peptide (OGP)

OGP, a 14-amino acid H4 histone-related peptide, improves the anabolic effect of osteocyte by activating the intracellular Gi-protein-MAPK pathway [181]. This peptide is abundant in the culture medium of osteoblasts and can improve bone formation and bone mineral content through an anabolic effect [181,182]. OGP can regulate differentiation and proliferation of osteoprogenitor cells, osteocalcin secretion, collagen, and matrix mineralization *in vitro* [183]. *In vivo*, OGP was found to regulate the expression of osteogenetic factors, such as TGF-β1, TGF-β2, TGF-β3, FGF-2 and IGF-1 [184]. OGP is also used as a potential factor to enhance bone repair process in animal models. Shuqiang *et al*. [185] studied an OGP along with a PLGA scaffold and used it to treat a 1.5-cm local bone defects in rabbit model. The results showed a higher bone mineral content and faster bone repair response in the experimental group. OGP could promote osteoblast and fibroblast activity, increase bone mineral mass, and prevent bone loss. Moreover, this peptide can used to treat osteoporosis and bone fracture formation (Table 3).

Parathyroidhormone (PTH)-derived peptides

The main function of PTH is to regulate the metabolism homeostasis of calcium and phosphorus in mammals. $PTH_{1–34}$ is a curtate peptide with 34 amino acids that retains its bioactivity. Furthermore, $PTH_{1–34}$ is used to the systemic treatment for osteoporosis and bone formation regulation. Moreover, multiple cell types are involved in the mechanism of $PTH_{1–34}$ stimulating bone tissue repair. For example, previous studies demonstrated that PTH activated the survival signaling pathway of osteoblasts and osteoclasts, and that delay of osteoblast apoptosis mainly contributed to the increased osteoblast number in rat models [186]. The effect of $PTH_{1–34}$ therapy on skeletal repair has been demonstrated. Manabe *et al*. [187] showed that the treatment group had superior biomechanical properties on fracture healing in a femoral fracture model. To evaluate the effects of $PTH_{1–34}$ on bone regeneration, Komatsu demonstrated that $PTH_{1–34}$ equally promoted endochondral or intramembranous osteogenesis of femoral fracture defects in the rat models [188]. Additionally, using local $PTH_{1–34}$ therapy combined with bone scaffolds has been proposed to promote bone regeneration. Jung *et al*. demonstrated that the polyethylene glycol-based matrix containing PTH_{1-34} peptides strengthened bone repair and accelerated the whole process in a canine mandible defect with a 1.5 mm gap [189]. Systemic PTH_{1-34} treatments directly affect the gene expression of MSCs and osteoblasts. Kaback *et al*. revealed that $PTH_{1–34}$ could enhance MSC differentiation into osteoblasts via osterix and Runx2 expression [190]. To date, PTH_{1-34} therapy is widely used in treating osteoporotic fractures in elderly populations and has demonstrated many benefits, such as acceleration of bone repair processes, reduction of the cost and required hospital time, and the rebooting of bone formation process in delayed unions or non-unions. A randomized and controlled trial of PTH_{1-34} showed that PTH_{1-34} therapy decreased the healing time from 9.1 weeks to 7.4 weeks versus placebo in the distal radius fracture of postmenopausal women patients [191]. Reynolds *et al.* reported a case that $PTH_{1–34}$ effectively improved union fractures between the broken ends of tibial for 4.5 months after injury [192]. At present, the capacity of PTH_{1-34} to promote bone repair and regeneration has been verified, and its application

combined with new bone scaffold materials will become the focus of future BTE research (Table 3).

Heparin-binding peptides

Growth factors and molecular proteins have been used to enhance bone repair and formation. However, these molecules have limited application because of their short half-time, unstable bioactivity, and immune-related side effects. Therefore, heparin-binding motifs may be a suitable alternative to avoid these shortcomings and maintain bioactivity of osteogenetic factors in bone regeneration. Heparin-binding peptides can combine with heparin sulfate (HS)-proteoglycans (PG) on the target cell surface, leading to a decrease in immune-related reactions and initiating bone regeneration signaling pathways. In particular, these peptides are involved in FGF-mediated and BMP-mediated differentiation of MSCs to osteoblasts [193]. For instance, Choi *et al*. synthesized a peptide consistent with BMP-4 residues (heparin-binding domain) and showed that this peptide could induce osteoblastic differentiation by regulating the ERK signaling pathway [194]. Interestingly, when combined with gel scaffold alone, the heparin-binding peptides can still participate in bone formation. Therefore, their assembly with alginate gel matrix can be used as a novel bioactive construct for bone regeneration (Table 3).

Chemical molecules

Similar to osteoinductive peptides, several chemical molecules with small sizes of less than 1000 Da (or 1 kDa) can also participate in bone repair processes. These molecules are usually uncharged and hydrophobic in nature and can permeate cellular membranes through direct fusion (Table 4). These molecules induce the differentiation of osteoprogenitor cells by initiating signaling cascades that lead to gene transcription and expression. Signaling transduction systems, such as cyclic monophosphate (cAMP)/PKA, Hh, BMP/Smad, MAPK, and Wnt, have demonstrated their functions in regulating the osteogenic process [198].

Statins

Statins, which are also known as HMG-CoA reductase inhibitors, are routinely used as lipid lowering medications. In addition, statins also act as the activator of BMP signaling pathways, and this activation induces ectopic bone formation in a rat model of subcutaneous implantations [199]. Simvastatin can induce the mineralization of new bone trabecula and upregulate the expression of BMP-2 and other bone-related elements in human osteoblasts [200]. Several other compounds from the statin family have also been investigated. For instance, fluvastatin can improve the osteointegration and increase mineralized bone volume when locally applied on fractured tibiae of rats [201]. Moreover, statins have also been used to treat the long bone defects *in vivo* models. Moriyama *et al*. observed significantly increased bone callus formation after simvastatin injection at the defect site of rat tibias [201]. These new properties of statins have drawn considerable attention in BTE field. However, the therapeutic dosage has not been effectively demonstrated, and the adverse effects of high dose statin remain unknown.

Phenamil

Phenamil, which is a derivative of diuretic amiloride, activates many different signaling pathways, particularly the BMP/Smad signaling activator. This small molecule supports cell proliferation, promotes ALP activity, and facilitates bone matrix mineralization in osteoblast precursor MC3T3-E1 cell lines [202]. Furthermore, the application of phenamil may increase the level of integrin α5 and phosphorylation of c-AMP. These effects can promote the signaling cascade that is necessary for osteogenesis [202]. Phenamil has an additive effect on bone regeneration when combined with BMP-2 and BMP-7. This molecule has also been observed to induce the expression of the Tr3b protein and downregulates Smurf1 (negative regulator of BMP signaling) expression. This process leads to increased levels of BMP signal transducer Smad1/5/8 [203]. Although phenamil has not been

Table 3 Peptides types in BTE

Peptide	Cells	Effects	References
OGP	Rat mesenchymal stem cell	Increase of osteogenic markers in osteoinductive medium and new bone formation *in vivo*	[195]
PTH	MC3T3-E1	Increased hydrazine-bisphosphonates affinity to bone and improved hydrazine bisphosphonates interaction with osteoblastic cells in basal medium	[196]
Heparin-binding peptide	Human mesenchymal cells	Increase of mineralization in osteoinductive medium	[197]

Table 4 Different kinds of small osteoinductive molecules

Small molecule	Drug delivery system	*In vitro*/*in vivo* osteogenesis	References
Phenamil	2D/3D scaffold	Enhanced proliferation of MC3T3 cells, increased ALP expression and enhanced mineralization	[202]
Oxysterols	Hydrogel/sponge/3D scaffold	Oxysterol-21 promoted bone formation in the callus and increased mechanical stability of lumber vertebral segments	[216]
Purmorphamine	Bone adhesive composite beads	Activation of Hh pathway	[222]
FTY720	Bone autografts/3D scaffold	Coated allografts promoted new bone formation with significant increases in mechanical properties when compared to the controls	[221]
Simvastatin	3D scaffold/porous bone Cement/ hydrogel	New bone formation was observed after scaffold implantation in tibial defect model. *In vivo* osteogenesis were observed when loaded scaffolds were combined with MSCs cell sheet	[223,224]
TH (helioxanthin derivative)	Tetrapod-shaped granules	Promote osteoblastic differentiation in mouse MC3T3-E1 cells	[225]
FK506	3D scaffold	Evidenced by the ectopic bone formation in subcutaneous location	[226]

investigated clinically, animal experiments have already verified that phenamil has a positive effect on stimulating bone formation and preventing osteoporosis [204].

Helioxanthin derivatives

Similar to phenamil, helioxanthin derivatives also play an important role in the BMP/Smad signaling pathway. Zhao *et al*. demonstrated that helioxanthin derivatives induced osteoblast-like MC3T3-E1 cell linages, as well as promoted primary osteoblasts and embryonic stem cells to osteoblastic cells [205]. The osteogenic potential of helioxanthin derivatives was verified when bone scaffolds containing helioxanthin derivatives were implanted into a rat model of calvarial defects. This study showed that ectopic bone formation occurred within 4 weeks [206,207].

Purmorphamine

Purmorphamine, which is a purine derivative, induces osteogenesis in mouse embryonic mesoderm fibroblasts [27,28]. The Hedgehog (Hh) signaling pathway is linked to osteogenesis *in vitro*, and purmophamine is a known agonist of this important pathway [209,210]. This role is further confirmed by *in vivo* studies. Wu *et al*. demonstrated that trisubstituted purines could increase ALP expression in mouse embryonic fibroblasts [208]. Further studies demonstrated that CaP beads soaked in purmophamine solution increased the percentage of bone trabecular mineralization [211,212]. However, no interrelated preclinical studies to verify the function of this molecule *in vivo* have been reported.

Oxysterols

Oxysterols, which are derived from cholesterol oxidation, control osteoblast differentiation not only via the Hh pathway but also by activating the Wnt signaling pathway. Oxysterols promote the synthesis and deposition of CaP in ECM scaffolds [213]. Significant enhancement in bone formation and regeneration was also observed after treatment with oxysterols on the calvarial defects and spine fusion defects in rats [214,215]. Hence, oxysterols can play an important role in many biological processes, such as osteogenesis, but the human applications need further research to confirm this hypothesis.

Adenosine cAMP analogs

The cAMP analogs perform their osteogenic properties via the cAMP/PKA signaling pathway. Siddappa *et al*. showed that the small cAMP analog dibutyryl cyclic-AMP (db-cAMP) could upregulate the expression of ALP in BMSCs and have a positive result on mineralization [216]. Another cAMP analog, 6-Bnz-cAMP, also has osteogenic inductive properties. Laurencin *et al*. studied this molecule and discovered that the expressions of ALP, RunX2, osteocalcin, and osteopontin were upregulated in MC3T3-E1 cells in mice [217]. 8-Br-cAMP could also enhance both angiogenesis and osteogenesis *in vitro*, and only this molecule stimulated VEGF secretion, which resulted in increased bone matrix mineralization [218].

FTY720

FTY720, which is a natural product-derived immune

suppressant, plays a crucial role in many cellular processes, such as stimulating EC migration and proliferation, enhancing neovascularization during bone healing, and reducing osteoclast deposition on bone fractures [219]. When this molecule was applied to bone scaffolds in animal fracture models, the test subjects showed better bone formation compared with single scaffold. For instance, Petrie *et al*. implanted a poly (D, L-lactic-co-glycolic acid) PLGA scaffold loaded with FTY720 into a critical rat calvarial defect and showed that this scaffold maintained the highest amount of new vessel formation compared with the control group [220]. Furthermore, when using allograft implants coated with FTY720, the implant showed prominent new bone formation with high density of vessel networks [221].

Other small molecules

Small interfering RNAs (siRNAs)

Gene therapy aims to deliver genetic material to change the downstream gene expression during bone formation processes. Introducing genetic molecules into osteocytes is the most efficient process for either reducing bone resorption or promoting cell proliferation and differentiation toward an osteogenic lineage. The siRNAs have an innovative biological capability to silence the target gene, opening a new field for siRNA therapy in BTE [208]. For example, using siRNA to knock down the expression of BMP inhibitors has been widely explored to improve bone formation. One key problem to overcome is to ensure the safety and effectiveness of delivering siRNA to target cells. Hence, robust drug delivery systems (DDSs) are essential (Table 5).

DDSs have two major categories that are used to successfully deliver the siRNA into osteoblasts, namely polymer-based and lipid-based systems [227]. PLGA has been explored as a viable DDS for delivering siRNA, and siRNA-PLGA compounds have facilitated bone regeneration. For example, Hong *et al*. fabricated PLGA microparticles to encapsulate siRNA that provided a sustained release *in vitro* over 40 days [228]. Moreover, due to the receptor activator of nuclear factor κB (RANK) receptors responsible for activating osteoclastic gene expression, Wang *et al.* explored the use of PLGA microparticle-encapsulated RANK-siRNA compounds to enhance bone formation [229]. Semaphorin 4d (Sema4d), which is an osteoblast-osteoclast communication messenger, is released in osteoclasts to reduce bone mineralization. Therefore, the application of PLGA- encapsulated siRNA was shown to silence the gene of Semad4 as a treatment for osteoporosis in mouse mandibular alveolar models [230]. Similar to the polymer-based system, the lipid-based DDSs are also used to transfect cells with siRNA to enhance osteogenic differentiation.

At present, siRNA-based therapies have been paid attention, and the efficiency shows promise in bone tissue regeneration. However, siRNA therapies present several disadvantages. First, the process may silence genes which are non-target sequences. In addition, an immune-related response is typically caused by either the siRNA or the DDS. Finally, the function of normal miRNA may be influenced because of the interference of endogenous RNA [232]. Despite these drawbacks, several methods can enhance the efficacy of siRNA therapy. These techniques include the incorporation of osteogenic factors (such as BMPS and VEGF) with siRNA to improve bone regeneration processes [233].

Moreover, the growth factors, peptides, and small molecules have not reached clinical application although they have shown evident osteoinductive potential. Molecules, such as purmorphamine, have been extensively investigated *in vitro*. However, relevant studies using these molecules have not been conducted in bone fracture models, and many osteogenic molecules tested in rodent models have led to inconclusive results. Small molecules have undoubted potential for bone repair, but the major limitation of nonspecific adverse effects still exist. These limitations can be overcome by using new scaffold biomaterials, matrices, and DDSs. Hence, advances in material design should be a cornerstone for the development of small molecule applications.

Integration of BTE constituents

The ideal scenario for scaffold-based BTE is to combine the scaffold with seed cells to form a bioengineered construct that can facilitate bone repair. The characteristics

Table 5 Different types of other small molecules for BTE

Gene of interest	Delivery system	Cell/animal model	Function	References
Gucocorticoid receptor (GR)	Polymer	Human bone marrow	Successful siRNA delivery and release for up to 40 days	[228]
RANK	Polymer	Murine osteoclast precursor cells	Inhibition of bone resorption due to RANK expression knockdown	[229]
Semaphorin 4d	Polymeric nanoparticles	Ovriaectomy in mice	Decreased bone loss resulted from osteoporosis	[231]

of the biomaterial scaffolds, such as chemical composition, surface topography, 3D microenvironments, and abundant cell-adhesion sites, provide the essential structure and substrates for bone regeneration and mediate the cell adhesion and specific cell activity to participate in bone mineral, collagen deposition, osteogenic proliferation, and differentiation [234,235]. Therefore, regulating and controlling the parameters of scaffold design may affect the cell-scaffold interactions while directly influencing the result of bone tissue regeneration.

The microporosity of the scaffold has been considered as an essential parameter in bone tissue growth. Scaffold pore structure (such as pore size and interconnectivity) is an essential consideration in scaffold fabrication. The optimal pore size influences migration and adhesion of cells in diverse shapes and sizes, while significantly affecting cell morphology and phenotypic expression [236]. The pore size of the scaffold determines the density of ligands and the number of adhesion factors available for cell attachment, as well as influences the activity of vascular smooth muscle cells that are crucial in vascularization [237]. Integrins, such as adhesive ligands and number of receptors, can mediate transduction signal cascades and induce cell–ECM and cell–cell adhesion formations as a result of ECM ligand binding in receptor conformations [238]. Interconnectivity provides the basic microstructure and is essential for natural cellular migration, growth, and nutrient delivery.

To better mimic the natural bone, nanostructures (such as nanoparticles or nanofibers) have been designed to intensify the mechanical properties of the scaffolds for load bearing. Nanocomposites have been applied to produce osteoinductive scaffolds by increasing the surface area and volume ratios, enhancing the surface roughness, and changing the nano-topography. These nanostructured scaffolds can influence stem cell behavior, such as cell adhesion, viability, proliferation, and osteogenic differentiation, and therefore stimulate new bone regeneration [14,239].

BTE includes many physical and biochemical signals for new bone to integrate with the host bone tissue. Considering the osteogenesis and angiogenesis processes during bone formation, fabrication of scaffolds, which control the delivery of bioactive factors, is certainly a new strategy to spatiotemporally regulate improvements in tissue regeneration. However, single biomolecules are usually insufficient to promote complex bone regeneration processes, with multiple and sequential delivery systems finding promise as potentially mimicking complex clinical situations [240]. Growth factors and cytokines, such as BMPs, TGF-β, and IGFs, have been combined with 3D scaffolds to influence cell signaling processes that can systemically change the levels of cell gene expression. In addition, new techniques have been applied to incorporate seed cells into scaffolds, such as being encapsulated within bifabricated 3D scaffolds to solve time-consuming, inefficient, operator-dependent, and cell random distribution problems. Although several advances have been achieved toward the delivery of growth factor and cell-based methods, these scaffolds generally lack command over the arrangement of the engineered well-aligned vascular networks in 3D space. This phenomenon result in randomly organized networks. However, fabrication approaches to engineer pre-vascularized scaffolds with pre-defined microvascular network have been developed to overcome this issue. Pre-vascularized scaffolds use advanced biomanufacturing techniques to construct the functional capillaries within the scaffolds to improve cell viability and tissue growth. Therefore, novel strategies for nanostructured scaffold combined with bioactivators may play significant roles in fabricating suitable BTE scaffolds.

Challenges and perspectives

An ideal bone tissue-engineered model should possess characteristics similar to the gold standard of autografts without any general restrictions. BTE is supposed to provide the most desirable features, such as biological safety, long-term viability, low donor injury and morbidity, reasonable cost, no size restrictions, and superior osteogenic and angiogenic properties. However, BTE is facing significant challenges that should be resolved before this gold standard is achieved in a clinical setting.

First, more effective methods of cell isolation, cultural seeding, and osteogenic differentiation should be developed to improve the survival rate of stem cells and osteogenic cells during the preimplantation period. In terms of biological safety, tumorigenic potential of various MSCs is a clear concern. Thus, available regulated processes of cell differentiation should be implemented to avoid this issue. In addition, the mechanism by which stem cells achieve the regenerative process remains ambiguous because of the combination of osteogenic scaffolds and growth factors. Thus, further studies should be performed to explore the underlying mechanisms and signaling pathways of the interactions among the seed cells, biomolecules, and biological scaffolds for better clinical applications.

In recent years, many 3D porous scaffolds have achieved significant progress to provide the extracellular microenvironment. However, new domains should be explored toward better imitation of natural bone regeneration by integrating angiogenesis and osteogenesis procedures. Simultaneously, these “smart” scaffolds should have the ability to spatiotemporally regulate the release of growth factors to effectively stimulate bone tissue formation and decrease nonspecific adverse effects. Despite the biocompatibility and biodegradability of the scaffolds, the majority of these materials can serve as host antibodies and

induce dangerous immune responses. Furthermore, the vascularity of the synthesized scaffold is critical, and the regenerative process could be disturbed because of the lack of sufficient and timely vascularization. Thus, new strategies of bioengineering vascularized scaffolds are essential to rapidly promote the survival of the implanted cells, reduce tissue necrosis and fibrosis in early stage.

Several other challenges, such as ethical issues, uncertain long-term quality, potential complication of patients, and the translation into clinical applications, should also be investigated to effectively translate these scaffolds into clinical practice in the near future. Although a large number of bone regeneration strategies have been investigated, no clear optimal choice has been established.

Conclusions

We reviewed the recent advances in various types of stem cell selection and differentiation, cytokine biology and signaling mechanisms, scaffold fabrication, and applications relevant to bone regeneration. Although diverse combinations of scaffold are available, significant limitations, such as poor osteogenesis, inadequate vascularization, and low delivery of growth factors, still exist. Hence, current novel strategies and techniques should concentrate on osteogenesis of stem cells, promotive factor delivery, and 3D angiogenic scaffolds for successful bone regeneration and clinical translation.

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Compliance with ethics guidelines

Rui Shi, Yuelong Huang, Chi Ma, Chengai Wu, and Wei Tian declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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