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Review

Exploring calcium-free alternatives in endochondral bone repair tested on *In vivo* trials - A review

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ABSTRACT

Bone repair via endochondral ossification is a complex process for the critical size reparation of bone defects. Tissue engineering strategies are being developed as alternative treatments to autografts or allografts. Most approaches to bone regeneration involve the use of calcium composites. However, exploring calcium-free alternatives in endochondral bone repair has emerged as a promising way to contribute to bone healing. By analyzing researches from the last ten years, this review identifies the potential benefits of such alternatives compared to traditional calcium-based approaches. Understanding the impact of calcium-free alternatives on endochondral bone repair can have profound implications for orthopedic and regenerative medicine. This review evaluates the efficacy of calcium-free alternatives in endochondral bone repair through *in vivo* trials. The findings may guide future research to develop innovative strategies to improve endochondral bone repair without relying on calcium. Exploring alternative approaches may lead to the discovery of novel therapies that improve bone healing outcomes.

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Abbreviations: BTE, Bone Tissue Engineering; EO, Endochondral ossification; TCP, Tricalcium phosphate; HAP, Hydroxyapatite; ACP, amorphous calcium phosphate; OCP, Octacalcium phosphate; DCBM, Decellularized Cancellous Bone Matrix; ASC, Adipose Stromal Cells; BMP-6, Bone Morphogenetic Protein 6; BMP-2, Bone Morphogenetic Protein 2; SSC, Skeletal Stromal Cells; VEGF, Vascular Endothelial Growth Factor; SAA, Salvianic acid; HA, Hyaluronic acid; GAG, Glycosaminoglycans; DBM, Decellularized Bone Matrix; EDC, 1-ethyl-3-carbodiimide hydrochloride; NHS, N-Hydrosuccinimide; bFGF, Basic Fibroblast Growth Factor; PCL, Poly(ε-caprolactone); PEG, Poly-ethylene glycol; PGA, Poly-glycolic acid; MMP, Metalloproteinases; PGS, poly(glycerol sebacate); PLLA, Poly-L-Lactic acid; MSCs, Mesenchymal stromal cells; BMSCs, Bone marrow stromal cells; hBMSCs, human Bone marrow stromal cells; rBMSCs, rat Bone marrow stromal cells; HUVECs, Human umbilical cord vein endothelial cells; Ad-MSCs, Exogenous adipose-derived mesenchymal stem cells; hOAs, human osteoarthritic articular chondrocytes; PDCs, human periosteum-derived cells; iPSCs, human pluripotent stem cells; MAG, Mangiferin; LLP2A-Ale, LLP2A alendronate; HyC, hypertrophic cartilage; hHyC-ECM, Devitalized human hypertrophic cartilage extracellular matrix; PDCM, Particulate decellularized cartilage matrix; PRP, Platelet-rich plasma; Vac-OS, Vacuum-assisted osmotic shock; EBR, Endochondral bone regeneration; PTHrP, hap-loinsufficiency of endogenous parathyroid hormone-related peptide; MEW, Melt Electrowriting.

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1. Introduction

Critical-size bone defects resulting from trauma, congenital disorders, and tissue resection require more than two million bone grafts annually [1]. These defects exceed the self-healing capacity of natural bone and can significantly affect a patient's appearance and musculoskeletal function [2]. Autogenous bone grafting is the clinical "gold standard" due to its exceptional immunocompatibility and inherent osteoinductive, osteoconductive, and osteogenic properties. However, it has limitations such as donor site morbidity, limited graft availability, and decreased regenerative potential with donor age [3]. Allografting is less common due to the risk of immune rejection and disease transmission [4], while xenografting raises concerns such as infection and rejection [5]. In addition, these treatments require multiple surgeries, bone fixation devices, and slow regeneration processes. This can sometimes lead to improper graft integration, hindering healing [6].

As an alternative, bone tissue engineering (BTE) strategies have transformed bone healing and regeneration treatments. BTE is an interdisciplinary field that combines engineering and life science principles to develop bioartificial bone tissue to repair bone defects caused by trauma or congenital disorders and to restore, maintain, or improve tissue function [7]. Strategies developed in BTE utilize biomaterials, extracellular matrices, osteogenic cells, growth factors, and gene therapy [8]. Various biomaterials, including metals, natural or synthetic polymers, and ceramics, including calcium compounds such as tricalcium phosphate (TCP), hydroxyapatite (HAP), amorphous calcium phosphate (ACP), and octacalcium phosphate (OCP), have shown promise in bone repair applications [8–12]. In addition, the integration of natural and synthetic materials with cell therapies and bioactive molecules has produced remarkable results in large bone defect models through chondrocyte hypertrophy, cartilage matrix template, mineral deposition, and bone formation [13].

Historically, BTE approaches have focused on scaffold design using calcium compounds as the primary osteoinductive composite, a topic that has been extensively researched and reviewed [13–15]. However, materials other than calcium-based composites have gradually become the focus of attention in bone tissue engineering constructs. Research on non-calcium-based composites has led to the development of innovative scaffold materials and fabrication techniques that may improve outcomes in BTE [16]. The use of non-calcium-based composites reduces the risk of unwanted calcification or mineralization in surrounding tissues, a common problem with calcium-based materials. In addition, non-calcium composites may offer better biocompatibility for certain applications, which could reduce immune responses or adverse reactions in the body [16]. However, non-calcium-based composites also have limitations. These composites may not have the osteoconductive properties of calcium-based materials, such as hydroxyapatite, which are essential for promoting bone growth and integration. In addition, different non-calcium-based composites may have different degradation rates in the body, potentially compromising the overall structural integrity and longevity of the scaffold [17]. The interactions between non-calcium-based composites and host tissues may also be more complex and less predictable than those with calcium-based materials, requiring additional research and optimization for successful integration [18].

This review analyzes and presents new approaches developed in the last decade to repair severe bone injuries without relying on calcium composites in the context of endochondral bone repair. The review explores non-calcium-based composites prepared with natural and synthetic polymers, metals, bioactive molecules, growth factors, and stem cells as potential substitutes for traditional calcium-based composites in bone tissue engineering. This review aims to assess the efficacy and potential impact of non-calcium alternatives on bone regeneration by analyzing the current research landscape and the results of these innovative approaches. It will also provide insight into the future potential of these emerging strategies and their impact on bone tissue engineering. Fig. 1 summarizes the major groups of materials used in the fabrication of non-calcium based scaffolds or constructs for endochondral ossification research that have been evaluated *in vivo*.

1.1. Natural materials

Natural materials have emerged as promising alternatives in tissue engineering for repairing large bone defects, due to inherent properties such as high biocompatibility, close resemblance to

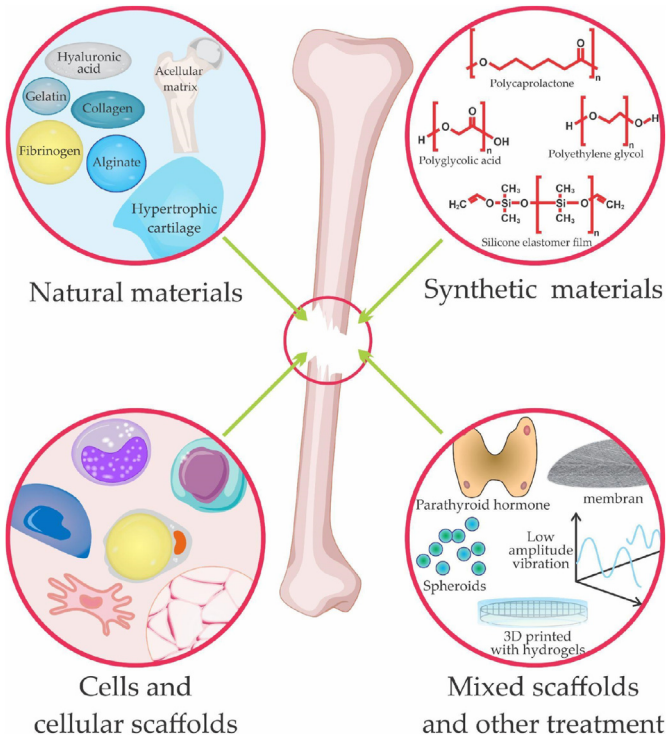

Fig. 1. Strategies used and evaluated *in vitro* and *in vivo* for bone repair via endochondral ossification without calcium composites.

natural tissues, and gradual degradation over time. Gelatin, fibrinogen, collagen, and alginate, as well as bone and cartilage from autologous, allogeneic, or xenogeneic sources, are used to create hydrogels or scaffolds for bone tissue engineering and EO promotion that do not contain calcium compounds. These materials and methods have been tested in relevant environments with promising results; some of which are shown in Fig. 2.

Some examples of the scaffolds mentioned that have been tested include ground cortical bone powder with bone marrow stem cells (BMSCs) in a fibrinogen gel [22], or constructs containing MSCs micropellets and fibrin that have been shown to stimulate bone formation in injectable bone graft substitutes [23], and cancellous bone that has been decellularized and partially demineralized [24] in rabbit femur and murine models. These results showed decreased fibrotic tissue in the affected area, new trabeculae formation, vascularization, and significant bone formation or calcified cartilage due to EO [22,24].

Collagen has been extensively studied for its role in bone repair because it is a major component of cartilage and the bone's extracellular matrix [25,26]. As a result, collagen has been tested with different geometric structures and cell types, including adipose stromal cells (ASCs), skeletal stromal cells (SSC's), and mesenchymal stromal cells (MSCs), to take advantage of order to capitalize on their combined properties for EO-mediated bone repair. Collagen with geometric structures based on oriented channels demonstrated greater potential for bone repair by EO than random collagen structures that did not contain growth factors or cells within the scaffold [27]. Similarly, ASCs and collagen formed a cartilaginous matrix *in vitro* that was then implanted subcutaneously in mice, resulting in a hypertrophic cartilage matrix via endochondral ossification, with potential use in long bone repair [28]. Collagen and SSC's have also been evaluated for their ability to form bone via EO in an ectopic approach in mice, showing great potential for bone repair [29]. The MSCs approach involves chondrogenic differentiation within the collagen scaffold, consisting in the combination of BMSC's suspension with collagen type I and a reconstitution buffer, subsequently seeded with chondrogenic differentiation medium for 3 weeks and hypertrophic differentiation medium for another 2 weeks for posterior subcutaneous implantation. This method induces endochondral ossification in mice, resulting in the formation of blood vessels and the deposition of various types of collagens, including types I, II, and X (Fig. 2a) [19].

Collagen-based scaffolds loaded with bioactive molecules or growth factors are a promising approach to develop effective bone tissue engineering techniques [30]. Bioactive molecules such as angiostatin [31], bone morphogenetic protein 2 (BMP-2), vascular endothelial growth factor (VEGF) [32], oxysterol [33], and salvianic acid [34] have been shown to promote EO alongside collagen sponges and matrices with unidirectional architecture, decrease the expression of inflammatory and angiogenic genes, and increase the expression of EO markers, thereby accelerating the transition from cartilage to bone. These collagen scaffolds containing bioactive molecules have been tested in mice, rats and rabbits.

Gelatin, a collagen derivative, is biodegradable and biocompatible, with lower antigenicity than collagen, making it an ideal material for bone repair [35]. In terms of EO, gelatin crosslinked

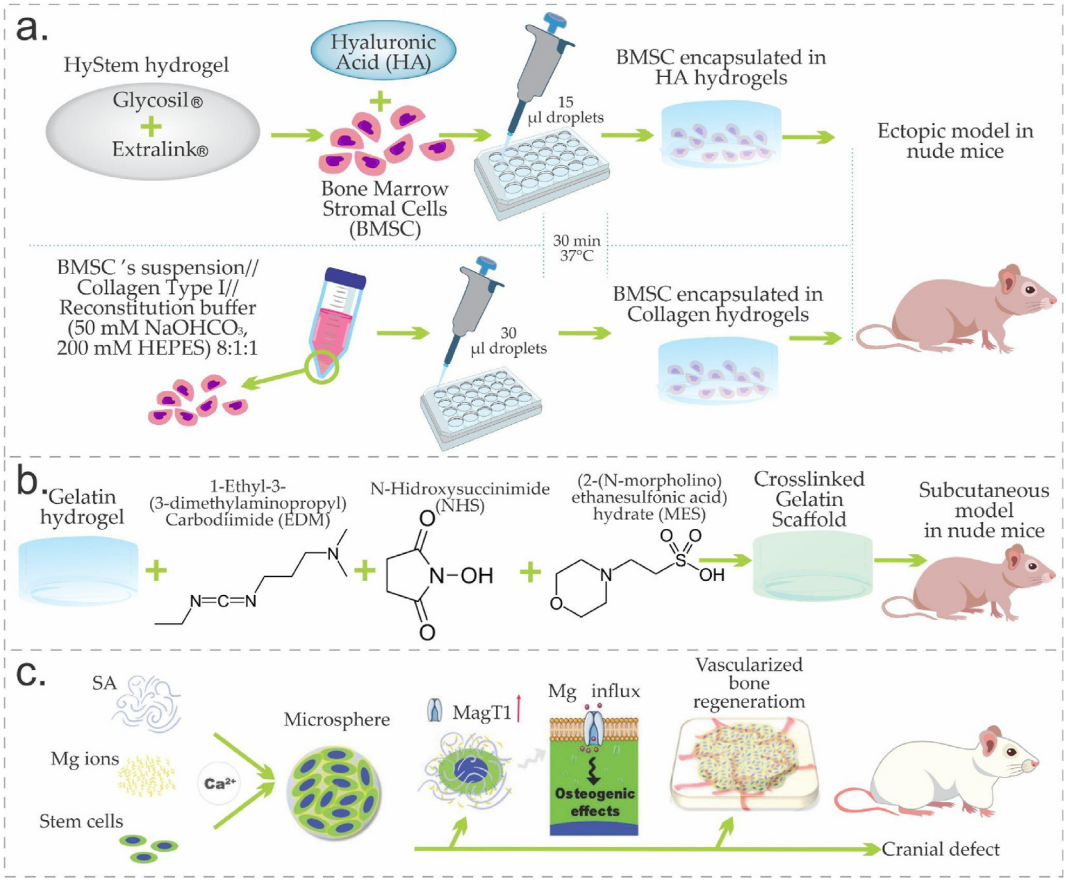

Fig. 2. Some strategies using natural materials for bone repair by EO in *in vivo* models. a. Fabrication method of hyaluronic acid (HA) and collagen hydrogels with encapsulated BMSCs to promote endochondral ossification (adapted from Ref. [19]). b. Materials needed to obtain cross-linked gelatin scaffold by carbodiimide chemistry (EDC and NHS) with a reconstitution buffer (MES). (Adapted from Ref. [20]). c. Schematic construction of the Mg-enriched 3D culture system promoting MagT1 expression for bone regeneration and evaluation in rat cranial defects. (adapted from Ref. [21]).

with 1-ethyl-3-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) via carbodiimide chemistry produced varying degrees of stiffness and allowed for chondrogenic and osteogenic differentiation of mouse mesenchymal stem cell line, C3H10T1/2, and pre-osteoblastic MC3T3-E1 cells over time *in vitro*. For *in vivo* approach, BMSC's were seeded on these gelatin scaffolds, resulting in increased stem cell-mediated trabecular bone formation in stiffer scaffolds tested in C57BJL/6 mice (Fig. 2b) [20].

Gelatin, like collagen, can be loaded with various growth factors and bioactive molecules that enhance bone repair capacity via endochondral ossification. Gelatin hydrogels loaded with basic fibroblast growth factor (bFGF) significantly improved bone healing in a femoral fracture model in C57BL/6 mice [36]. BMP-2 and transforming growth factor beta 1 (TGF-β1) promote bone and blood vessel formation [37], induce EO by accelerating bone healing and remodeling [38], and stimulate BMSCs to chondrogenic differentiation [39]. In addition, BMP-2 combined with chondroitin sulfate in a gelatin matrix improves the regenerative potential of EO in bone healing in mice [40].

Alginate is another versatile biomaterial that facilitates cell encapsulation and growth factor delivery, making it a promising candidate for guiding endochondral bone formation [41]. This material allows for the incorporation of a variety of substances such as peptides, kartogenin, magnesium, or cells to enhance EO. For example, peptides such as RGD (arginine, glycine, and aspartic acid) and QK (VEGF mimicking peptide) in combination with kartogenin were found to play a key role in regulating cell behavior for osteochondral defect repair [42]. Magnesium-enriched alginate microspheres promoted vascularized bone growth by mimicking key developmental processes by upregulating the expression of magnesium transporter-1 (MagT1), a selective magnesium (Mg) transporter, in a mouse embryo model and enhance formation of vascularized bone in cranial defects in rats (Fig. 2c) [21]. Alternatively, ASC-derived alginate constructs demonstrated superior bone-forming capacity, chondrogenesis, and vascularization in mice after four weeks of chondrogenic induction and eight weeks of subcutaneous implantation [43].

Biomaterials have been tested for their ability to promote EO after ectopic implantation in nude mice. For example, hyaluronic acid (HA) (HyStem hydrogel) loaded with Bone Marrow mesenchymal stromal cells (BMSCs) has been shown to exhibit complete integration in all biomodels evaluated, producing bone with vascularization and marrow development between fused grafts in ectopic model in nude mice (Fig. 2a) [19].

Another novel material for bone repair by EO is Adiscaf, a fractionated human adipose tissue designed to differentiate adipose stromal cells (ASCs) and facilitate cartilage formation. It contains glycosaminoglycans (GAGs) and type II collagen, enabling the potential for bone tissue formation by EO in an ectopic approach in nude mice [44] in the treatment of long bone defects in nude rats [45,46]. Adiscaf has shown higher GAG production, superior bone formation and vascularization than decellularized bone matrices (DBM).

Using natural materials in bone tissue engineering for repair by EO without using calcium compounds is an excellent way to develop new technologies for repairing long bone defects. However, it is important to note that these materials need to be functionalized and modified to achieve better mechanical behavior and properties that contribute to bone vascularization, formation, and remodeling. A summary of different fabrication methods and *in vivo* evaluations is presented in Table 1.

1.2. Synthetic materials

Synthetic materials have emerged as innovative solutions in orthopedics and bone repair, offering promising alternatives to natural materials. Synthetic materials used in bone repair can be divided into metal materials, polymer materials, and composite materials. Synthetic polymers and metallic biomaterials combined with bioactive molecules are novel strategies for bone scaffold fabrication and/or advanced therapy design. In addition, synthetic materials overcome several limitations, including the availability of natural materials and immunological reactions. Likewise, synthetic scaffolds prepared by various techniques such as electrospinning, 3D printing, and hydrogels have osteoinductive and osteoconductive properties and superior physical and mechanical properties that resemble native bone. In addition, incorporating bioactive molecules induces strategic modifications that enhance physical, chemical, and biological properties to direct site-specific tissue formation while maintaining biomimetic architectures. These modified structures have the potential to support tissue healing and regeneration processes and promote faster and more effective recovery. Fig. 3 offers insight into the process of design, fabrication, and *in vitro* and *in vivo* testing of diverse synthetic materials, using techniques such as 3D printing, hydrogel design, and 3D melting electrospinning as alternatives to bone tissue engineering strategies for critical defects without use of calcium compounds. Different designs that have been tested provide a similar native architecture, contributing to an optimal environment for bone healing and regeneration. These materials, coupled with cell therapy strategies and/or growth factors that have been incorporated, have shown outstanding results in critical-sized bone defect healing.

1.2.1. Polymers

Polymeric materials offer novel solutions for bone tissue engineering, particularly for the repair of critical bone defects by endochondral ossification (EO). These materials exhibit high biocompatibility and gradual degradation, which are essential for successful bone repair. A variety of compounds such as polycaprolactone (PCL), polylactic acid (PLA), poly(L-lactide-co-epsilon-caprolactone) (poly(LA-co-CL)), polyglycolic acid (PGA), and polyethylene glycol (PEG) have been used to obtain fibrous scaffolds and hydrogels that have been fine-tuned and tested in relevant environments for bone tissue engineering applications. Table 2 summarizes the strategies using these polymers and their results in bone tissue engineering. This section explores the role of polymers in advancing bone regeneration and highlights their potential to address critical challenges in bone defect repair.

Polycaprolactone (PCL) is a widely studied polyester in many tissue engineering applications. This polymer has been extensively used to fabricate electrospun PCL nanofiber scaffolds that could mimic the fibrous microarchitecture of bone extracellular matrix; however, they are limited by their mechanical properties. In this sense, porosity optimization and enhanced mechanical properties have been tested and shown to be successful approaches as flexible 3D scaffolds that support chondrogenic differentiation of stem cells and promote endochondral bone formation in long bone defects (Fig. 3a) [47,50].

Accordingly, PCL and its copolymers have been used to develop new scaffolds through polymer casting, 3D printing, and hydrogel-like structures as alternatives for bone-critical defects. For example, polymer casting of poly(L-lactide-co-epsilon-caprolactone) (poly(LA-co-CL)) into a cylindrical architecture has been reported to be a more effective method of inducing endochondral ossification in polymeric bone scaffolds, thereby improving the healing of bone-critical femoral defects in 12-week-old rat models. Similarly, organized 3D architecture has been achieved through additive manufacturing. 3D-printed cylindrical fibrin PCL scaffolds were designed to support mesenchymal cell adhesion and promote chondrogenic or hypertrophic differentiation, thereby achieving

Table 1
Strategies using natural materials evaluated *in vivo*.

Material	Loading or complement	Fabrication	*In vivo* model	Key findings	Ref
Fibrinogen gel	Cortical bone powder and Bone marrow stromal cells (BMSC's)	Cryoprecipitation for fibrinogen gel and BMSC's differentiated osteogenic lineage.	Femur defects in rabbits	Osteogenic BMSCs, combined with the natural scaffold, promoted bone maturity and decreased fibrosis.	[22]
	Mesenchymal Stromal Cells (MSC's)	Crosslinking of thrombin and fibrinogen loaded with MSC's	Ectopic model in nude mice	Micropellet-fibrin-MSC's constructs, with a shorter *in vitro* priming time, showed comparable bone formation to standard MSC's pellets *in vivo*.	[23]
Decellularized and demineralized cancellous bone (DCBM)	N/A	Decellularization of cancellous bone and demineralization by ultrasound	4 mm defect in medial femoral epicondyles in New Zealand white rabbits.	More newly formed trabeculae, vessels, and endochondral bone were observed during early-stage bone repair *in vivo* in groups treated with specific mineralized DCBM.	[24]
Collagen	N/A	Directional freezing	5 mm femur defect in rats	Collagen with a channel-like pore architecture can control cell recruitment and tissue patterning for bone healing.	[27]
	Adipose Stromal cells (ASC's)	Type I collagen and Bone Morphogenetic Protein 6 (BMP-6).	Ectopic model in nude mice	ASC's can generate ectopic bone through ECO in the presence of collagen type I foam, similar to BMSC.	[28]
	Skeletal stromal cells (SSC's) and B6.Cg-Tg(ACTb-eGFP) mice embryos.	Encapsulation of SSC in collagen capsules	Ectopic model in NMRI nu/nu mice	Collagen with encapsulated SSCs delivers biochemical cues that guide their differentiation towards the osteogenic lineage	[29]
	Angiostatin	Loading Mesenchymal Stromal Cells (MSC's) onto collagen I scaffolds with or without angiostatin	Ectopic model in Lewis rats.	Angiostatin reduced inflammation and vascularization but did not induce fibrocartilage formation in subcutaneously implanted collagen scaffolds with or without MSC in rats.	[31]
	Vascular Endothelial Growth Factor (VEGF) and Bone Morphogenetic Protein 2 (BMP-2)	Isolation of bovine collagen, and fabricating uni- and multidirectional scaffolds by freezing the collagen dispersion, followed by lyophilization. VEGF and BMP-2 were loaded with cell culture medium.	Muscular pockets in Wistar rats	Unidirectional collagen scaffolds had superior mechanical properties and liquid uptake capacity compared to multidirectional ones. Additionally, both types of scaffolds supported cell growth and osteoblastic differentiation *in vitro* and supported bone formation when loaded with BMP-2 or BMP-2 + VEGF, with pore orientation affecting the osteogenic process.	[32]
	Oxysterol (Oxy133)	Loading Oxy133 on collagen sponges	Spinal fusion in Lewis rats	Oxy133 induces significant expression of osteogenic markers and shows potential as an osteogenic molecule with improved synthesis and fusion time.	[33]
	Salvianic acid (SAA)	Loading a compound of SAA and a bone targeting liposome (SAA-BTL) on collagen sponges.	Radius defect in New Zealand rabbit	SAA-BTL-incorporated collagen sponges significantly stimulated bone formation in the nonunion defect rabbit model. Also, increased the expression of P-HDAC3, collagen II, RUNX2, VEGFA, and osteocalcin.	[34]
Type I collagen and hyaluronic acid (HA)	Bone marrow stromal cells (BMSC's)	Resuspend the BMSCs in collagen or hyaluronic acid to create hydrogels containing the cells.	Ectopic model in nude mice	*In vivo*, both collagen and HA constructs promoted vascularization, endochondral bone formation, and bone marrow development. However, HA constructs demonstrated superior fusion ability, forming integrated bone tissues with evidence of vascularization and marrow development between fused grafts.	[19]
Gelatin	N/A	Crosslinking of gelatin scaffolds by carbodiimide chemistry	Ectopic model in C57BJL/6 mice	EDC treatment enhances trabecular bone formation; and the high mechanical strength of 3D scaffolds promotes stem cell-mediated bone regeneration through endochondral ossification.	[20]

(*continued on next page*)

Table 1 (*continued*)

Material	Loading or complement	Fabrication	*In vivo* model	Key findings	Ref
	Basic fibroblast growth factor (bFGF)	Crosslinking of gelatin scaffolds with glutaraldehyde	Critical size femur defect on C57BL/6 mice	Hydrogels incorporating bFGF showed significantly stronger bone regeneration compared to bFGF solutions.	[36]
	Transforming growth factor beta 1 (TGF-β1), bone morphogenetic protein 2 (BMP-2), Bone marrow stromal cells (BMSC's)	Soaking gelatin hydrogels in BMP-2 solution.	Femoral defects in rabbits and Rowett nude (RNU) rats.	Promotion of bones and blood vessel formation in a femur defect model in a New Zealand rabbit.	[37]
		Encapsulating TGF-β1, BMP-2 and BMSC's in gelatin microspheres.	Calvarial defect in Rowett nude (RNU) rats	Mesenchymal condensations induce bone formation based on morphogen presentation, with BMP-2 + TGF-β1 fully restoring mechanical function.	[38]
Alginate	RGD and QK peptides and kartogenin	Microscaffolds of alginate and RGD, loaded with Kartogenin, and a final layer of RGD and QK peptides. Crosslinking was made by carbodiimide chemistry.	Ectopic model in BALB/c nude mice	Alginate, RGD and QK microscaffold-hydrogel composites effectively accelerate bone growth and enhance stem cell behavior, and controlled release of kartogenin induces chondrocyte differentiation and hypertrophy for accelerated osteochondral repair.	[42]
	Magnesium	Mixed sodium alginate with BMSC's in a Mg enrichment culture medium.	Critical size cranial defect in Sprague Dawley rats	The Mg-enriched cell delivery vehicles promote osteogenic differentiation of stromal cells and lead to significant vascularized bone regeneration in rats with critical-sized cranial defects.	[21]
	Adipose Stromal cells (ASC's)	Loaded ASC's within alginate beads	Ectopic model in nude mice	Comparisons against ASC constructs in high-density pellets versus alginate bead hydrogels indicated that hydrogel culture may be a more promising method for bone tissue engineering via the endochondral pathway due to the exhibit better vascularization and higher cell retention.	[43]
Hypertrophic cartilage matrix	Adipose Stromal Cells (ASC's)	Culture of fractionated human liposuction for 3 weeks and posterior culture of ASC's to hypertrophic differentiation for 2 weeks.	Ectopic model in nude mice	Employing adipose tissue as a scaffold exhibited enhanced *in vitro* differentiation and superior *in vivo* performance in comparison to the conventional approach of isolating and expanding ASCs in monolayers.	[44]
		Decellularization of bovine juvenile trabecular bone and ASC's differentiated to chondrogenic lineage.	5 mm femur defect in RNU nude rats	Tissue-engineered bone grafts using hypertrophic chondrocytes led to enhanced bone deposition and bridged a higher number of defects compared to osteoblast grafts and acellular scaffolds.	[45]

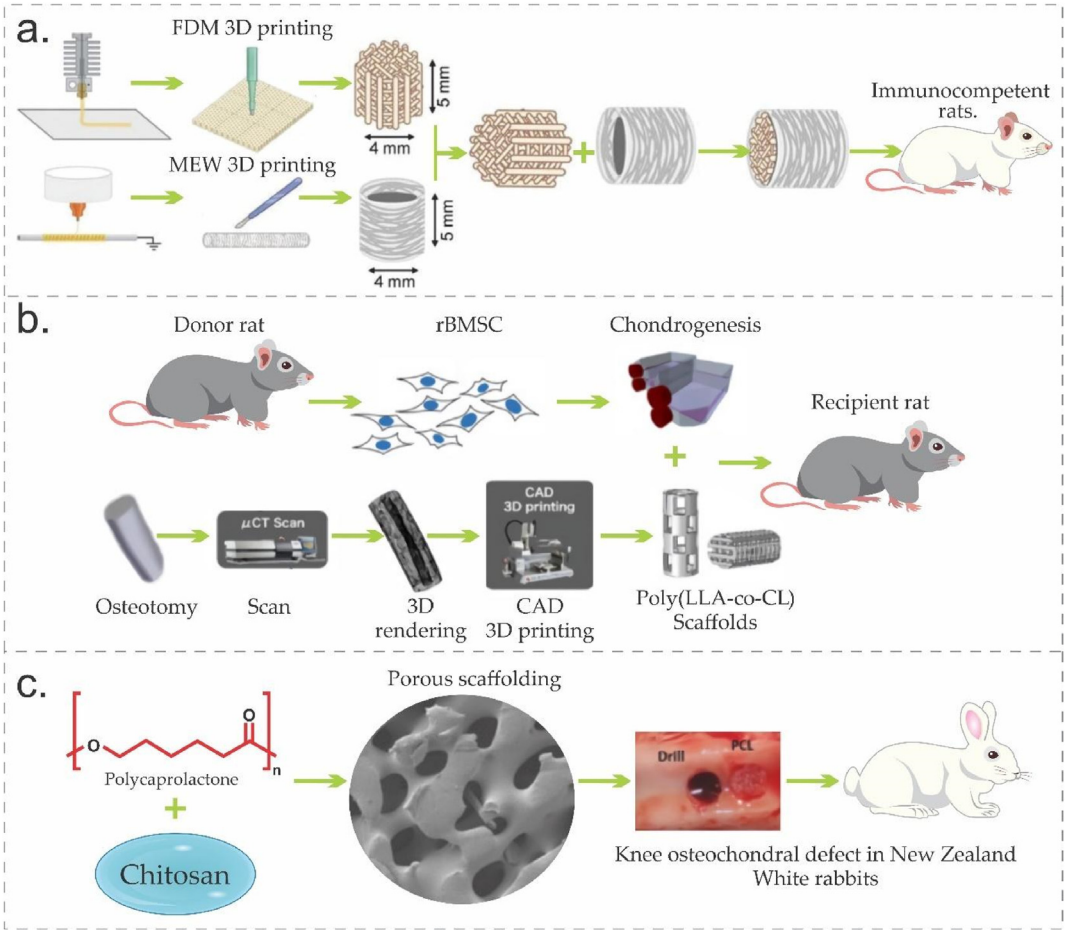

Fig. 3. Application of non-calcium compounds in bone tissue engineering approaches using synthetic biomaterials. a. Schematic representation of the 3D printed scaffold created using fused deposition modeling (FDM) and scaffold fabrication using Melt Electrowriting (MEW). The 3D scaffold was assembled by combining the inner implant core and the MEW membrane. The *in vivo* evaluation of the scaffold shows the functionalization and vascularization of the mimetic periosteum (Adapted from Ref. [47]). b. The tube scaffold is designed with a simulated medullary canal and has pores on its walls to stimulate vascularization. In contrast, the cylindrical scaffold is fabricated using a 0/90 lay-down pattern. Rat bone marrow-derived mesenchymal stem cells (rBMSC) were seeded onto printed scaffolds and underwent sequential chondrogenesis for 21 days. Afterward, they were implanted into the defects for a follow-up period of up to 15 weeks (Adapted from Ref. [48]). c. PCL scaffolds coated with chitosan to create a composite material, which was then evaluated for its effects on various tissue regeneration processes. (Adapted from Ref. [49]).

the initial stages of EO processes for large bone healing (Fig. 3b) [48]. Similarly, PCL hydrogel-like structures have been explored as alternatives to traditional scaffolds for mimicking hypertrophic cartilage microtissues. Polycaprolactone-based structures coated with chitosan and various cell lines, such as hBMSCs and human umbilical vein endothelial cells (HUVECs), have been used to achieve complete and efficient bone regeneration [52].

These studies have shown promising results of polycaprolactone scaffold approaches, such as ectopic bone formation, mineralized hypertrophic cartilage, and vascularization along the scaffold in various animal models, principally in the murine and lagomorph models, which are considered some of the most relevant models for BTE studies. In addition, low immune responses have been reported, suggesting scaffold integration and healing of critical-size bone defects throughout the EO in many cases (Fig. 3c) [49,52].

Besides PCL, PGA has been extensively studied and used in BTE applications. This thermoplastic polymer has been combined with different cell lines using porous scaffolds as a support structure and a cell therapy delivery vehicle. For example, 3D scaffolds enriched with BMSCs or chondrocytes have been developed to mimic endogenous bone-healing processes by promoting hypertrophic cartilage formation. Notably, preclinical rat models have shown vascularization in the scaffold, high-density mineralization, cell recruitment, and osteoclast activity throughout the implanted area, suggesting that endochondral processes were occurring [59,60].

Similarly, PEG is one of the most commonly used synthetic polymers for the development of degradable hydrogels. This specific structure was designed to mimic the characteristics of the extracellular matrix of cancellous bone. In this sense, proteins such as metalloproteinase (MMP) and thiolene alginate have been mixed with PEG, and stem cells or periosteum-derived cells are some of the most relevant approaches. These strategies support blood vessels and nerves in the graft, resulting in increased ectopic bone formation, density, and better structural incorporation of the implanted scaffold into the endogenous bone. Interestingly, these alternatives could overcome the lack of treatment availability and the osseointegration problems of traditional treatments [54,56].

Furthermore, elastomeric polymers have been used in BTE, which are desirable because they offer customizable properties amenable to bone regeneration. For example, poly(glycerol sebacate) (PGS) is considered one of the most promising materials for BTE because its stiffness can be tuned to match osteoid or immature tissue rather than mature bone. It has been shown that tailoring these specific mechanical properties is sufficient to induce mechanobiological responses that promote bone regeneration. This means that PGS is a promising material that needs to be thoroughly

Table 2
Studies in bone tissue engineering using synthetic polymeric biomaterials.

Material	Loading or complement	Fabrication	*In vivo* model	Results	Ref
PCL	BMP-2 expressing cells	Electrospinning and freeze- drying techniques	Subdermal implantation on nude mice	*In vitro* osteogenic differentiation was achieved on the microporous scaffold. Additionally, subdermal implantation of the cell-loaded scaffold showed cell infiltration, cartilage-like tissue formation, and hypertrophic cartilage matrixes.	[50]
	rhBMP-2	3D printing and melt electrospinning	5 mm femur defect on rats	Bone volume and density were achieved after 10 weeks of implantation on the femur defect, suggesting complete healing.	[47]
Poly(ethylene glycol) diacrylate	Human articular chondrocytes (hACs) & Human mesenchymal stem cells (hMSCs)	Photo Encapsulation in polymeric hydrogels	2-mm segmental defect by osteotomy in the mid-tibial diaphysis of immunocompromised mice	Cartilage grafts enhance bone regeneration, promoting highly vascularized new bone tissue.	[51]
Chitosan, PCL, fibrin, gelatin hyaluronic acid	HUVECs & hBMSCs	3D printing and bioprinting	Subcutaneous implantation on nude mice	Pre-vascularized scaffold showed a higher potential, promoting higher mineralization and angiogenesis after 4 weeks prior subcutaneous implantation	[49,52]
PGA, hyaluronic acid, and fibrin	hBMSCs	Hydrogel	3,5 mm one defect on parietal lobes on transgenic mouse	*In vivo* study showed a high capacity for cell recruitment all over the hybrid matrix, leading to new bone formation and highly vascularized structures through endochondral ossification processes.	[53]
PEG, MMP	mMSCs, adipose tissue pellets	Hydrogel	4 mm femoral defect on murine models	Metalloproteinase hydrogels showed increased new vessel formation and density, coupled with high nerve density, after 9 weeks. The scaffold was shown to induce early endochondral ossification and new bone formation.	[54]
Poly(L-lactide-co-epsilon-caprolactone) (poly(LA-co-CL))	Rat bone marrow mesenchymal stem cells (rBMSC), pre-differentiated *in vitro* into cartilage-forming chondrocytes	3D printing	Critical-sized (5 mm) femur defects on rats	Significant new bone was formed inside and in the periphery of the implanted scaffold. Also, bone marrow space was reported with connective tissue, adipocytes, and mononuclear cells.	[48]
Silicone	Periosteal strips	Silicone elastomer sheeting	2,54 cm Mid-diaphysis bone defect on sheeps	Substitutes loaded with periosteal strips, proved to be significantly more efficient, inducing ectopic bone formation and bone regeneration within the muscle.	[55]
Thiol-ene alginate	3D Bioprinting	Hydrogel	Subcutaneous implantation on mice	Ectopic bone formation was evidenced on the implanted scaffold. This approach induced nodules of mineralized tissue.	[56]
PGS	rBMSCs	Casting and particulate leaching	16 mm Ulna defect on rabbits	Runx2 and Osteocalcin gene expression were increased in the PGS scaffold. Moreover, complete defect bridging was achieved after 4 weeks.	[57]
PLLA	VEGF	Core-shell electrospinning	Rat 5 mm diameter calvarial defect	Membranous porous scaffolds completely repaired the defect zone and formed a periosteum-like structure. Bone density and hyaline-like cartilage were found, indicating endochondral ossification repairing processes.	[58]

tested [57]. Similarly, a silicone elastomer film was used as a barrier membrane to mimic the key structural and functional properties of native periosteal tissue, including elastin, collagen, and progenitor cells. These results suggest that the elastomeric film generates bone tissue primarily through endochondral mechanisms [55].

Overall, the reported findings on elastomeric polymer scaffolds have made them promising alternatives for BTE. Nevertheless, the lack of follow-up studies and more robust results indicate that this novel approach requires extensive research that could lead to specific bone repair and regeneration mechanisms when using these materials in scaffold design.

On the other hand, scaffold design has focused on more than just creating a support structure or delivering cell therapy. The incorporation of bioactive molecules such as VEGF, BMP-2, TGF-β3 and IL-8 has gained prominence as these factors have been shown to promote faster and more efficient healing of critical bone defects. For example, PEG hydrogels encapsulating BMSCs and chondrocytes have been loaded with BMP-2 and TGF-β3 to enhance cellular response. Similarly, bioactive glass and poly(L-lactic acid) (PLLA) electrospun scaffolds have been used as bioactive molecule delivery systems for interleukin-8 (IL-8), BMP-2, and VEGF. These studies have concluded that the incorporation of these molecules into scaffolds is a novel approach to designing bone healing strategies. The sustained release of these factors leads to the development of endochondral bone, forming osteochondral components that could potentially lead to endochondral ossification processes. Although promising results have been achieved, process optimization and encapsulation efficiency must be considered [51,58,61].

In summary, several approaches to bone tissue engineering have been undertaken in recent years using synthetic materials and moving beyond calcium compounds. Studies have shown how different strategies have led to a new understanding of bone repair processes and how this mechanism can be used to induce early stages of endochondral ossification, healing and repair of critical bone defects.

Table 3
Metallic scaffolds that have been evaluated for *in vivo* endochondral bone repair.

Material	Loading or complement	Fabrication	*In vivo* model	Results	Ref
Titanium	N/A	3D printing	4 cm tibial defect on sheeps	Bone formation was observed, and complete bone bridging was achieved.	[62]
			Ovine lumbar fusion	Cell migration to the scaffold leads to cancellous bone formation. Osseointegration was achieved, and endochondral ossification was evident on the titanium scaffold.	[63]
CoCrMo	N/A	3D printing	Tibial defects on rabbits	Fibrocartilaginous tissue and a large/dense area of interconnected trabeculae. Additionally, angiogenesis was confirmed by the presence of multiple blood vessels in the trabecular area of the scaffold.	[64]

1.2.2. Metallic scaffolds

Metallic materials such as titanium and titanium alloys have become useful in orthopedic and bone replacement therapies due to their superior mechanical properties. Known for their remarkable strength and biocompatibility, they have been tested in relevant environments that have positioned them not only as replacement materials but also as repair and bone tissue engineering materials. In this sense, lagomorph and ovine models have been extensively used to study them due to their anatomical similarity to humans in this specific tissue. In addition, the locomotion, bone healing similarity, and vascularization comparability of these models have established them as gold standard models. Table 3 shows the metallic materials and their corresponding *in vivo* results.

Recently, versatile additive manufacturing technology has been used with superior metal/polymer-based filaments, causing 3D printed metallic scaffolds to take particular relevance due to their matching mechanical properties and optimizing pore size, emphasizing the importance of mechanobiological stimulation in critical-size bone defect healing. Therefore, titanium-printed scaffolds have shown superior mechanical properties and osteoinductive and osteoconductive potential even more than novel materials, including PEEK. These characteristics have been successfully applied in preclinical ovine models with excellent results, mechanically supporting bone structures and enhancing tissue regeneration, indicating EO processes [62,63]. In addition, 3D-printed metallic alloys have been studied. For example, lagomorph models were used for carrying out trials of the effects of UV photofunctionalization of a CoCrMo alloy to improve its bioactivity for bone formation, revealing that photofunctionalized CoCrMo scaffolds exhibited superior bone formation and integration compared to untreated implants [64]. This highlights the potential of UV photofunctionalization to enhance the bioactivity of orthopedic materials.

1.3. Cells and cellular scaffolds

In recent years, cells and cell-derived biological matrices have been widely used in tissue engineering solutions to heal critical defects in long bones. Preclinical studies have shown that these tissue engineering strategies are a promising source of therapies for regeneration of large bone defects by guiding endogenous bone formation, mineralization, and critical defect bridging.

1.3.1. Cell approaches

A major focus of tissue engineering research has been using cells for bone repair via endochondral ossification. Mesenchymal stromal cells (MSCs) have been investigated for their potential to promote bone regeneration via endochondral ossification. This section reviews various applications and recent advances in cellular approaches to BTE. The discussion focuses on the changing landscape of cell-based strategies, highlighting their potential impact and advances in addressing critical bone defects through *in vivo* evaluations. Table 4 summarizes cell-based strategies tested for their efficacy in bone repair through endochondral ossification, demonstrating the transformative potential of cellular interventions in bone regeneration. In recent years, cells and cell-derived biological matrices have been widely used in tissue engineering solutions to heal critical defects in long bones. Preclinical studies have shown that these tissue-engineering strategies are a promising source of therapies for large bone defect regeneration by guiding endogenous bone formation, mineralization, and critical defect bridging.

MSCs play a critical role in BTE due to their multipotent nature and ability to differentiate into multiple cell types. They offer the potential for tissue repair and regeneration in regenerative medicine applications. BMSCs are particularly attractive due to their immunomodulatory properties, self-renewal and high proliferative capacity. These cells can differentiate into multiple lineages, including osteogenic, adipogenic, and chondrogenic pathways, making them valuable for various tissue engineering approaches [65]. Various studies have consistently demonstrated the ability of BMSCs to differentiate into chondrocytes and promote bone formation *in vivo* through endochondral ossification (ECO), making them an attractive candidate for bone regeneration and repair of critical defects [73,74]. Fig. 4 shows different methods that use BMSC for bone repair through endochondral ossification that have been demonstrated *in vivo*.

The epigenetic profile of BMSCs has been identified as a critical factor influencing their ability to mature into functional hypertrophic cartilage and facilitate bone defect healing via endochondral ossification [75,76]. In particular, the induction of chondrogenic and osteogenic pathways in these cells accelerates bone regeneration via endochondral ossification. For example, cartilage-like tissue with a calcified bone layer promotes healing, as demonstrated in a severe combined immunodeficiency mouse calvarial defect model (Fig. 4a) [77]. Furthermore, studies have shown that supplementation of BMSCs with bioactive substances such as dioscin and mangiferin (MAG) or their integration into the extracellular matrix can significantly improve bone formation via endochondral ossification in mouse models mimicking endochondral ossification (Fig. 4b, c, and 4d) [66–68].

Therapeutic applications of BMSCs go beyond direct cellular treatments. BMSC-derived exosomes have been shown to be important in promoting fracture repair in mouse models. These nanovesicles carry bioactive molecules that can modulate cellular processes and promote tissue healing [78]. This is achieved by stimulating osteogenesis and angiogenesis, highlighting the osteogenic potential of BMSCs through paracrine signaling mechanisms (Fig. 4e) [69]. However, further research is needed to optimize transplantation protocols and to exploit the paracrine signaling pathways of BMSCs mediated by exosomes or extracellular matrix (ECM) components. This will enable the translation of these

Table 4
Strategies using cells evaluated *in vivo* for repairing critical bone defects.

Cell type	Strategy	Model	Key findings	Ref
BMSC	Chondrogenic and Osteogenic induction	Immunodeficiency mouse calvarial defect model	Cells differentiated into osteogenic cells provided bone matrix proteins to reconstruct the bone defect.	[65]
	Combined with Dioscin	Drill-hole injury in mice subchondral bone on the distal end of femur	Treatment of dioscin increased collagen type X expression and promoted the hypertrophic differentiation of BMSCs	[66]
	BMSC-derived chondrocytes planted on demineralized bone matrix with or without Mangiferin (MAG)	Middle femoral defect model in mice	MAG-treated, BMSC-based grafts have better osteogenesis in a mouse bone-defect model	[67]
	Integrated into their ECM	Ectopic bone formation on immunodeficient male mice	Constructs undergo N-cadherin-mediated condensation and subsequent chondrogenesis, mimicking endochondral ossification	[68]
	BMSC-derived exosomes	Transverse femoral shaft fracture on CD9−/− mice	MSC exosomes stimulate osteogenesis and angiogenesis for bone tissue repair process	[69]
Ad-MSCs	Combined with LLP2A alendronate (LLP2A-Ale)	Mid-femur fracture in osterix-mCherry (Osx-mCherry) male and female reporter mice	This combination increased endochondral bone formation and enhanced callus maturation compared to LLP2A-Ale alone	[70]
hOA	Hypertrophic cartilage grafts	Segmental defects unilaterally at the tibial middiaphysis of immunocompromised mice	hOA can be transformed into endochondral tissues that are able to integrate with host bone, undergo vascularization, and heal critical-size long-bone defects in mice.	[59]
PDCs	Microaggregates with BMP-2	Tibia defect in immunodeficient mice	Vascularization, hypertrophic chondrocyte production, and endochondral ossification	[71]
iPSCs	Cartilaginous Organoids	Critical size long bone (tibia) defects of NMRI nu/nu mice	Cartilaginous organoids successfully bridged critical size long bone defects	[72]

findings into effective clinical interventions for bone abnormalities and fractures.

In addition to BMSCs, other cell sources such as Ad-MSCs, hOAs, PDCs, and iPSCs have demonstrated promising therapeutic potential for healing bone defects when combined with osteoinductive factors. The ability of these cells to generate hypertrophic cartilage is critical to their ability to heal damage through endochondral processes. For example, Ad-MSCs have effectively enhanced femur fracture repair in murine models when co-administered with LLP2A alendronate (LLP2A-Ale). This improved callus maturation and enhanced endochondral bone formation compared to LLP2A-Ale treatment alone [70]. Similarly, hOAs have demonstrated the ability to produce hypertrophic cartilage grafts that can integrate with host bone, undergo ossification, and promote the healing of critical-sized bone lesions in immunocompromised mice through endochondral repair processes [59].

Additionally, PDCs in microaggregate form with BMP-2 have been shown to induce chondro-osteogenic lineages *in vitro*. Upon *in vivo* implantation, PDCs have been shown to promote vascularization, hypertrophic chondrocyte formation, and endochondral ossification in a critical-sized murine tibial lesion model [71]. On the other hand, iPSCs have successfully generated both cartilage and bone tissue *in vitro* and *in vivo*. The cartilage tissue derived from these cells has been shown to effectively promote bone healing in an orthotopic model [72]. This study highlights the potential of induced pluripotent stem cells for creating functional skeletal tissue intermediates. These findings show the therapeutic potential of various sources of MSCs and the importance of exploring new strategies to improve bone healing and regeneration.

1.3.2. Cartilaginous templates

Researchers have explored various cartilage template-based strategies to address graft integration and vascularization challenges in bone tissue engineering. These templates promote bone regeneration through endochondral ossification by serving as temporary biomimetic structures that imitate the natural process of bone formation and facilitate bone healing, particularly in critical-size bone defects. Cartilage templates use the principles of endochondral ossification to help chondrocytes differentiate into hypertrophic cartilage. This cartilage then undergoes mineralization and remodeling, leading to the formation of new bone tissue. This process resembles the developmental and repair sequences observed during embryonic development and fracture healing. Ultimately, this enhances bone regeneration outcomes aligned with the body's native bone healing mechanisms [79].

One innovative approach is utilizing cartilage tissue derived from stem cells to stimulate bone repair through endochondral ossification. This method replicates the natural processes observed during embryonic development and fracture repair, demonstrating the potential for promoting bone repair through tissue transformation in a mouse model of segmental tibial lesions [80]. The study demonstrates that chondrocytes can be transformed into osteoblasts by activating the pluripotent transcription factor Oct4A. Additionally, endothelial cells can mineralize cartilage explants by secreting one BMP or providing a conditioned medium. These findings advance the understanding of how stem cell-derived cartilage can contribute to bone regeneration.

Recent advances in generating hypertrophic cartilage (HyC) templates from mesenchymal stromal cells (MSCs) have shown promise for improving orthotopic bone healing outcomes. Devitalized human hypertrophic cartilage extracellular matrix (hHyC-ECM) has demonstrated superior efficacy in rabbit defect models compared to currently available clinical products [81]. This innovative approach highlights the potential of utilizing HyC templates derived from MSCs to enhance bone regeneration and repair in orthotopic settings.

Additionally, investigations into enhancing the osteogenic potential of engineered hypertrophic cartilage through the involvement of monocytes have yielded varied results [82]. Although initial studies aimed at improving bone healing through monocyte participation showed promise, subsequent research has reported mixed outcomes with limited effects on remodeling or invasion processes. These findings emphasize the challenge of regulating the osteogenic properties of engineered hypertrophic cartilage and the necessity for additional research to determine the best methods to maximize the regenerative potential of HyC templates in bone healing applications.

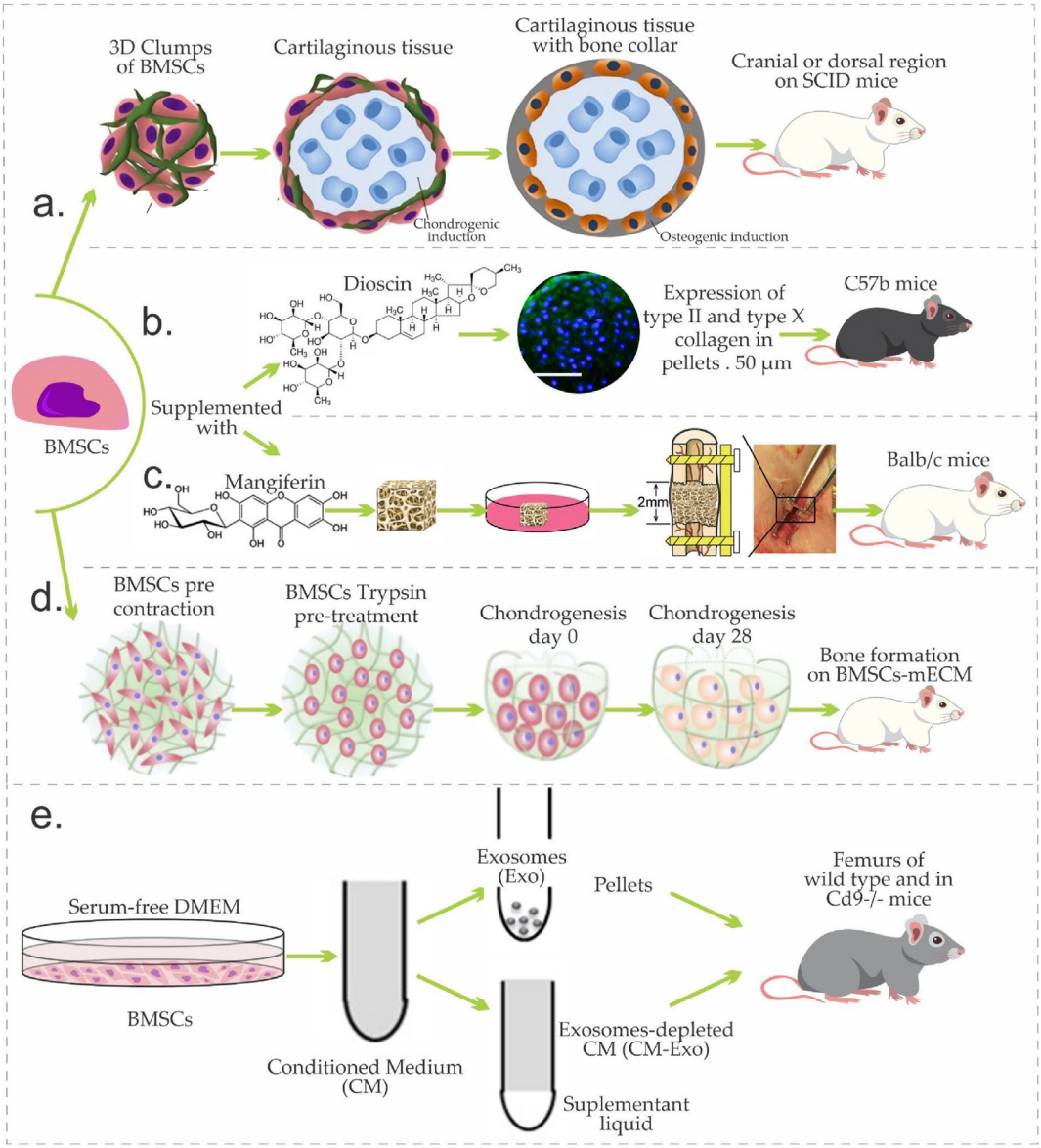

Fig. 4. Methods for bone repair by endochondral ossification studied *in vitro* and *in vivo* using BMSCs. a. C-MSCs were cultured in chondro-inductive and osteo-inductive mediums, resulting in cartilaginous tissue covered with a mineralized matrix layer. Treatment with only chondro-inductive medium resulted in cartilage with no mineralization. Transplantation of mineralized cartilaginous tissue induced rapid bone reconstruction via endochondral ossification in the mouse calvarial defect model. (adapted from Ref. [77]). b. BMSC pellets treated with Dioscin increase the expression of type II and type X collagen, promoting the osteochondrogenic differentiation of these cells. In animal models of subchondral bone fractures, Dioscin treatment enhance endochondral ossification (Adapted from Ref. [66]) c. The schematic diagram illustrates the process of endochondral ossification-based bone repair using BMSCs in combination with mangiferin. The cells were planted in the demineralized bone matrix for two weeks with MAG. The hypertrophic chondrocyte-based graft was implanted in a 2 mm bone defect in the middle of the femur in the mouse. This enhanced endochondral ossification-based bone repair. (Adapted from Ref. [67]). d. BMSCs embedded in their secreted ECM can form bone through endochondral ossification when given chondrogenic and osteogenic cues. A trypsin pre-treatment can alter cell morphology, enabling MSC-mECM constructs to undergo the condensation process and chondrogenesis. (Image adapted from Ref. [68]). e. Exosomes isolated from BMSC-conditioned medium (CM), supernatant without exosomes (CM-Exo), and an exosome pellet were injected into the fracture site. Delayed fracture healing in CD9−/− mice was rescued by injection of CM and exosomes. (adapted from Ref. [69]).

Similarly, decellularized scaffolds bioengineered and seeded with allogeneic BMSCs demonstrated osteoinductive properties. When implanted subcutaneously in mice, this type of scaffold induced vascularization and mineral accumulation, and half of the bone defects treated with hypertrophic cartilage scaffolds showed complete bridging [83].

Alternatively, injectable "cartilaginous" grafts incorporating particulate decellularized cartilage matrix, chondrogenically primed BMSCs bricks, and platelet-rich plasma gel have shown accelerated degradation of cartilage matrices and facilitated transformation processes, suggesting a promising avenue for generating vascularized bone tissue [84]. This approach showed that using BMSCs bricks in the constructs resulted in faster degradation of decellularized cartilage matrices, facilitating the transformation process. These findings suggest a promising method for generating vascularized bone tissue using injectable cartilaginous templates and BMSCs.

On the other hand, investigations into decellularized xenogenic hyaline cartilage xenograft matrices prepared using vacuum-assisted osmotic shock have demonstrated the preservation of structural integrity and differentiation potential for human chondrocytes and periosteum-derived cells [85]. This method preserves

Table 5
Mixed techniques and other methodologies applied in bone repair with their *in vivo* evaluation.

Material	Loading or complement	Fabrication	*In vivo* model	Key findings	Ref
Exogenous haploinsufficiency of endogenous parathyroid hormone-related peptide (PTHrP)	NA	Peptide extraction	Femur lessons on Wild-type and leptin receptor null Lepr(−/−) mice	Subcutaneous injection of PTHrP into Lepr(−/−) mice improves fracture repair by enhancing callus formation and accelerating cell transformation.	[87]
PCL	Cartilaginous spheroids from chondrogenic diff	Melt Electrowriting (MEW)	Ectopic and Orthotopic lesions on Immune compromised mice (Rj:NMRInu/nu)	Biohybrid lamellae, implanted subcutaneously for 4 weeks, mineralized (23 ± 3% MV/TV) and formed bone and bone marrow. Bone formation was observed when implanted in a critical-sized long bone defect in mice, although high variation between samples was detected.	[88]
	FPSCs & Chondrocytes BMSCs RGD- Alginate	Photoencapsulation on polymeric hydrogels	Osteochondral defect regeneration	The scaffolds facilitated the capture and fusion of cartilaginous spheroids with the potential to develop into bone.	[89]
Acceleration through whole body vibration	NA	Vibration-induced repair	Lessons from the eighth ribs on both sides in female Wistar Hanoverian rats	Low amplitude vibration (30-L) promotes chondrogenic differentiation and the expression of cartilage-related genes and may enhance fracture healing by promoting cartilage formation.	[90]
Osteoinductive autologous bone graft substitute (ABGS)	Recombinant human BMP6 (rhBMP6)	Bovine Achilles tendon-derived absorbable collagen sponge and bovine bone collagen as scaffold	Subcutaneous Rats implant and rabbit ulna segmental defect model.	The ABC scaffold within ABGS creates an environment conducive to bone formation, allowing the use of lower doses of rhBMP6 compared to other formulations. In addition, the newly formed bone remodels uniformly, integrating with the surrounding bone.	[91]

the structural and biological integrity of the cartilage xenograft matrix and allows differentiation of human chondrocytes and periosteum-derived cells. Similarly, devitalized cartilage constructs of allogeneic origin have demonstrated significant new bone production and defect-bridging capabilities after four weeks, indicating their potential for endochondral bone regeneration applications [86].

These diverse approaches utilizing cartilaginous templates or cartilaginous tissues underscore the multifaceted strategies available for enhancing bone regeneration outcomes and hold significant promise for future clinical applications in regenerative medicine.

1.4. Mixed scaffolds and other treatments

Innovative strategies have emerged to complement traditional approaches in the search for advanced techniques to repair endochondral bone. Among the interventions used are peptides, biohybrid films, microspheres, and autologous encapsulated and coagulated materials. They are all designed to enhance the effectiveness of bone healing mechanisms within endochondral repair. Table 5 and Fig. 5 provide an overview of the strategies and methodologies tested for bone defect repair, shedding light on the various approaches being investigated in bone regeneration.

Another approach to bone repair and fracture healing is the administration of parathyroid hormone-related peptide (PTHrP) (Fig. 5a). Studies have shown that PTHrP-derived peptides can enhance bone mass and strength, promote bone formation, and improve critical bone defect repair in various experimental models [92]. Moreover, exogenous PTHrP has been discovered to expedite fracture healing by promoting callus formation, upregulating osteoblastic gene and protein expression, and enhancing endochondral bone formation [87]. These findings regarding PTHrP's effects on bone tissue make it a promising candidate for promoting bone regeneration and addressing critical bone defects. Moreover, advancing enhanced PTH-related peptides provides a potential solution to overcome limitations associated with current treatments such as teriparatide, opening up new possibilities for improving bone metabolism and repair [93].

In a related development, researchers have investigated the combination of cartilage spheroids with electrically melted polycaprolactone membranes (MEW) as a means of creating cellular implants that induce bone formation *in vivo* (Fig. 5b) [88]. Seeding microspheroids onto MEW meshes results in improved expression of chondrogenic and prehypertrophic gene markers. Implantation of these biohybrid sheets has led to mineralization and bone formation, demonstrating their potential for treating critical-sized bone defects in murine models. The versatility of these scaffolds in bone repair applications is highlighted by their adaptability to varying defect sizes, as well as individual patient needs. This synergistic approach leverages the unique properties of each component to foster regenerative processes and facilitate bone regeneration through innovative scaffold design.

Furthermore, researchers have explored other mixed scaffold techniques, such as integrating 3D-printed polycaprolactone with alginate hydrogels and stem cells, to engineer constructs that mimic natural developmental processes for bone regeneration (Fig. 5c) [89]. These constructs have demonstrated the ability to induce endogenous endochondral ossification healing processes and promote the formation of cartilage resembling hyaline cartilage in caprine models. Moreover, the use of recombinant human bone morphogenetic protein 6 (rhBMP6) and autologous blood coagulum (ABC) as carriers for autologous bone graft substitutes has shown potential in enhancing the repair of critical bone defects and promoting bone formation in animal models (Fig. 5e) [91].

On the other hand, studies have demonstrated that vibration therapy can positively impact angiogenesis at the fracture site and surrounding muscles during the healing process (Fig. 5d) [94]. This therapy has been found to enhance bone callus formation, mineralization, and remodeling, ultimately accelerating osteoporotic fracture healing [95]. The mechanism through which vibration treatment influences fracture healing involves improving callus formation, mineralization, and remodeling processes [90].

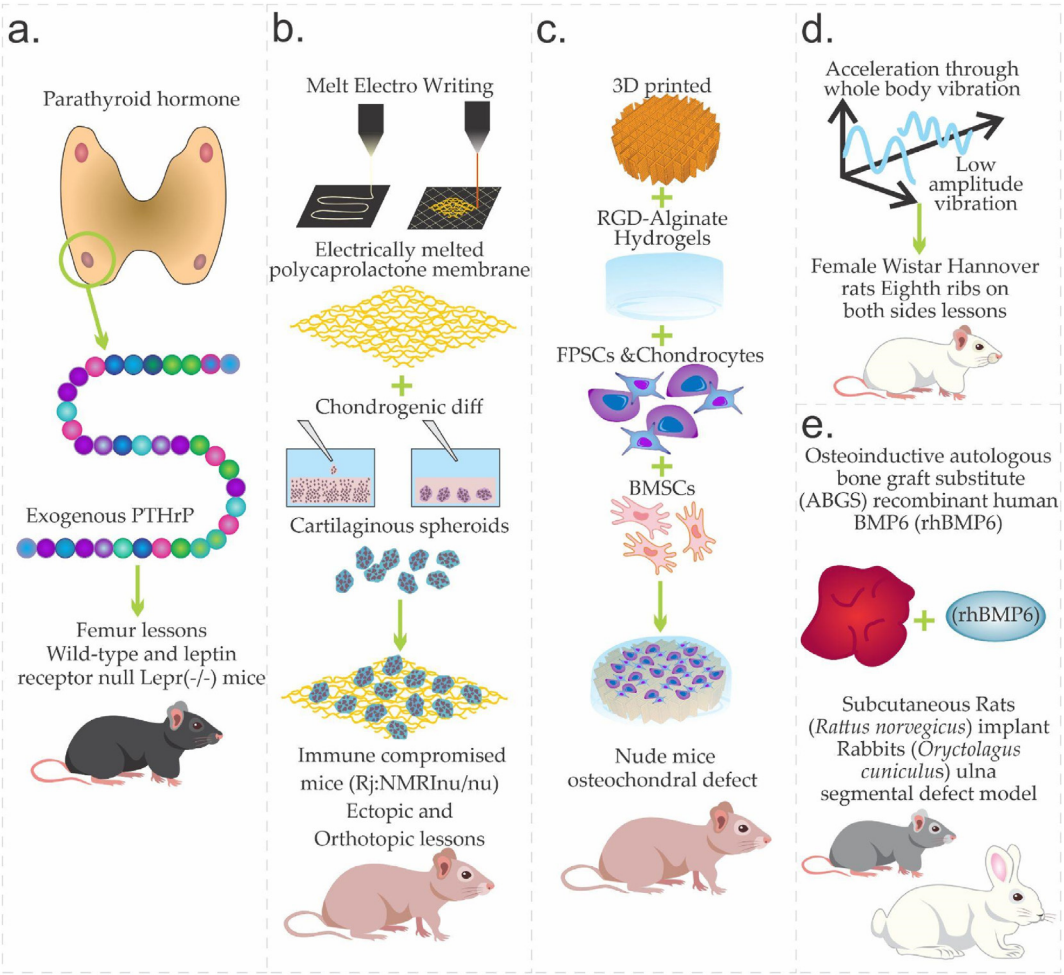

Fig. 5. Mixed and other techniques: a. graphic description of exogenous PTHRP tested in femur lesions of Lepr(−/−) mice (Adapted from Ref. [92]); b. Melt Electro Writing fabrication method, with spheroids from *in vitro* chondrogenic differentiation tested in immuno-compromised mice (Adapted from Ref. [88]); c. mix between 3D printed RGD-Alginate hydrogel functionalized with FPSC coculture and chondrocytes with BMSCs tested in osteochondral defects of nude mice (Adapted from Ref. [89]); d. Acceleration through whole-body vibration was tested in the number 8 ribs of Wistar Hannover rats (Adapted from Ref. [94]) e. Osteoinductive autologous bone graft substitute with recombinant human BMP6 was tested subcutaneously in *Rattus norvegicus* and *Oryctolagus cuniculus* ulna segmental defect (Adapted from Ref. [91]).

2. Conclusions and perspectives

Significant progress has been made in bone tissue engineering to develop effective regenerative strategies that use different biomaterials combined with cells and growth factors but without calcium components. Due to the complexity of biological systems, a multidisciplinary research approach is required to address key challenges. One of these challenges is the stimulation of the vasculature within scaffolds or constructs to ensure their survival and functional integrity after implantation. Tuning the mechanical properties of scaffolds to mimic natural tissues facilitates seamless integration with host tissues.

Recently, novel techniques inspired by embryonic processes have emerged and shown great promise. Advanced co-culture models incorporating different cell types are being developed to mimic the native stem cell microenvironment better. As our understanding of these mechanisms deepens, further advances are expected. At the same time, advances in material selection and manufacturing techniques are making commercially available products suitable for routine clinical use more feasible. This trend highlights the increasing viability of off-the-shelf solutions that can help researchers translate their discoveries into practical and effective treatments.

Achieving these goals requires a collaborative, multidisciplinary effort involving biologists, chemists, engineers, physicians, and orthopedic surgeons. Combining expertise across disciplines allows *in vitro* findings to be effectively translated into viable treatments. The future of bone regeneration therapy is bright because of the dedicated collaboration within the scientific community. This collaborative approach fosters innovation and accelerates the development of breakthrough solutions for complex bone defects.

Maintaining an ongoing dialogue and knowledge sharing among stakeholders is critical to advancing regenerative platforms and improving patient outcomes. The transformative potential of collaborative efforts is driving advances in bone tissue engineering, resulting in improved clinical applications and patient care.

Author contributions

William Cárdenas-Aguazaco, Adriana Lorena Lara-Bertrand, Leonadro Prieto-Abello, Nicolás Barreto-López and Ingrid Silva-Cote contributed significantly to the research, evaluation, writing, analysis, and interpretation of the consulted articles. Bernardo Camacho secured funding, and he critically reviewed the final manuscript. All authors have read and approved the published version of the manuscript.

Declaration of competing interest

Authors have no conflict of interest to declare.

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