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The rise of callus organoids for skeletal repair: Embedding developmental biology principles in technology-based tissue engineering

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Abstract

Treating critical-sized bone defects is challenging because successful repair relies on proper tissue transitions and timely vascular access, rather than on a single cell fate. Creating fully mature, defect-scale bone grafts *in vitro* remains constrained by scale, mass transport, and reproducibility, motivating strategies that deliver a programmed starting state and rely on *in vivo* progression. Organoid systems offer a useful paradigm in this context, as self-organizing microtissues can mimic developmental processes and produce consistent intermediate states. In this developmental engineering framework, callus organoids are cartilage-primed microtissues designed to follow an endochondral callus-to-bone path after implantation. This review synthesizes the mechanisms by which callus organoids are programmed to transition from chondrogenesis to hypertrophy, vascular invasion, ossification, and remodeling. It compares callus organoids to bone organoids and traditional scaffold-based bone tissue engineering, focusing on trajectory control, phase transitions, and timed integration with host transport and vascular systems. Key design variables include the endochondral potential of initial cells, the sequencing of biochemical and mechanical signals, and the timing of maturation and implantation to maintain vascular readiness. The review also discusses bioassembly and biofabrication in relation to diffusion limits and process-compatible potency assessment and quality attributes. Finally, donor variability, mass-transport limitations, incomplete multicellular complexity, and manufacturing standardization are key challenges driving priorities in perfusion and vascularization, architecture-informed fabrication, staged integration of vascular and immune components, and the development of extracellular matrix-based callus-mimetic templates. Overall, the emphasis shifts from building mature bone *in vitro* to manufacturing standardized callus-like building blocks whose potency is defined by their ability to execute orderly endochondral progression after implantation.

Keywords: Organoid; Callus organoid; Endochondral ossification; Developmental engineering; Critical-sized bone defects; Bioassembly; Biofabrication

1. Introduction

Bone fractures are common worldwide, with 178 million new cases in 2019.[1] While most heal, some result in delayed or non-union, with an overall risk of 1.9%, rising to 9% in certain age and site groups.[2] The challenge is most evident in critical-sized bone defects, which would not heal without intervention, and there is no universally accepted quantitative definition for these in the human field.[3] Beyond morbidity, non-union imposes a substantial

economic burden; contemporary claims analyses estimate a 2-year marginal cost on the order of USD 33,000–45,000 per case.[4] Autologous bone grafts are limited by volume and donor-site issues, while allografts raise safety and variability concerns.[5,6] These constraints define a translational space where "more bone" is not enough—what matters is how regeneration is initiated and sustained *in vivo*.

Despite advances in organoid technologies, replicating bone as a stable, functional organ *in vitro* is challenging due to its complex structure and reliance on perfusion-like transport, which complicates maturation and stability at clinical scales.[7,8] Dense three-dimensional (3D) tissues face diffusion limitations, and issues such as vascularization and protocol standardization hinder clinical translation in bone/cartilage organoids.[9] Moreover, the current implantation of bone-mimicking constructs has yielded suboptimal outcomes.[10–12] Therefore, for bone defect repair, engineering an implant that mimics the regenerative process rather than the fully mature bone could be more feasible.

Developmental engineering adopts a translational approach by mimicking key developmental programs, using lineage progression, phenotypic transitions, and spatiotemporal cues as adjustable parameters rather than relying on arbitrary combinations of materials and factors.[13,14] In skeletal repair, this framework is especially relevant to long-bone fracture healing, where endochondral ossification (ECO) plays a central role and a fracture callus forms with a transient cartilage phase before vascular invasion and bone formation.[15] For example, engineering a callus-like cartilage intermediate could support subsequent vascular invasion and ossification, while the *in vitro* phase is used to establish a defined maturation stage and chondrogenic-to-early-hypertrophic phenotype at the time of implantation.[13,14,16] This is a methodological approach to biomanufacturing living constructs: define the target tissue state, plan the transition path, and build quality controls that ensure batch-to-batch consistency.

A recent advance in bone tissue engineering (BTE) has led to the emergence of callus organoids, transient tissue constructs designed to repair bone defects via ECO.[17–19] This approach is based on previous developmental engineering research indicating that using a primed cartilage template yields more consistent bone formation than attempting to create mature bone solely *in vitro*.[20] Practically, the focus moves from producing bone *in vitro* to developing a temporary living tissue that evolves from callus to bone post-implantation.[21] Proof-of-concept studies support this developmental engineering logic, showing that a primed, callus-like intermediate can drive robust defect repair after implantation. For example, scaffold-free microaggregates derived from human periosteum-derived progenitor cells (hPDCs), after defined priming, can bridge a critical-sized murine long-bone defect through callus-mediated endochondral repair.[17] At the same time, embracing a transient, process-driven construct introduces new translational constraints: most notably donor-to-donor variability, diffusion-limited maturation, and the need for scalable, quality-controlled biomanufacturing, while still falling short of the full cellular complexity of fracture repair.[8,18] These advancements enable the shift of the central question from "Can we form bone?" to "Can we engineer a reproducible callus-to-bone transition that bridges defects at scale?"

This review first revisits bone formation and long-bone fracture repair through the lens of ECO, highlighting the transient fracture callus as a regenerative template particularly relevant to callus organoid engineering. We then position callus organoids within the broader skeletal tissue landscape by distinguishing them from bone organoids and classical tissue-engineering constructs. Next, we synthesize the biological constraints that matter most for engineering, including cell competence, the timing of differentiation cues, and maturation control, and connect these criteria to practical strategies for building and deploying callus organoids, including cell-source choice and biofabrication routes. Finally, we discuss key translational bottlenecks, such as donor variability and diffusion limits, and outline directions to address them, including improved vascularization, advanced fabrication workflows, and staged integration of additional tissue components for clinically relevant defect regeneration.

2. Fracture healing as a developmental blueprint for organoid design

2.1. Bone development

Skeletal development provides a reference program for how cartilage and bone are generated, patterned, and matured in a stage-dependent manner. There are two routes for bone development: intramembranous ossification forms bone directly from mesenchymal condensations, whereas ECO builds most of the axial and appendicular skeleton through a transient cartilage template.[22] In bones formed via ECO, proliferative chondrocytes within the cartilage anlage progressively undergo hypertrophic differentiation. This is followed by the advancement of an "ossification front," during which blood vessels invade the hypertrophic cartilage together with osteogenic, osteoclastic, and hematopoietic cells. As the process proceeds, the hypertrophic cartilage is resorbed and replaced by trabecular bone, while the marrow cavity is established at the primary ossification center.[22–25] This sequence is not a passive replacement process, because hypertrophic chondrocytes can actively contribute to bone formation by giving rise to osteoblast lineage cells in addition to recruiting vascular and osteogenic invasion.[26–28]

For instance, vascular endothelial growth factor (VEGF) produced by hypertrophic chondrocytes is a key trigger for capillary invasion and the coupled transition from cartilage remodeling to bone formation; blocking VEGF disrupts this handoff, but the process can be rescued when inhibition is relieved.[29] Importantly, this program is inherently modular and time-gated, suggesting that regenerative potency can emerge from controlled transitions rather than a static endpoint.[25]

From an engineering standpoint, the key to development is that cell competence and matrix context change across stages, and these shifts are actively instructive rather than incidental. The extracellular matrix (ECM) is not a passive scaffold: it is dynamically remodeled, encodes tissue-specific biochemical and biophysical cues, and can concentrate or present signaling factors to guide cell behavior and fate decisions.[30,31] In ECO, skeletal morphogenesis involves coordinated transitions between cellular programs and their microenvironments, such as chondrocyte maturation coupled with angiogenic invasion and osteogenic/hematopoietic recruitment.[15] The vasculature and immune system co-evolve with the matrix and progenitor states, facilitating orderly development.[15,32,33] Similarly, fracture repair is seen as a regenerative process that reactivates developmental patterns, rather than merely reconstructing bone as a static outcome.[34]

2.2. Endochondral ossification during fracture healing

In many long-bone fracture repairs, healing often follows a staged regenerative program in which inflammation, cartilage formation, vascular invasion, and bone remodeling are temporally coordinated (Figure 1).[15,35] After injury, disruption of local vasculature leads to a hematoma and a cytokine-rich inflammatory milieu that clears debris and recruits progenitors (hematoma phase).[15,34] These initial events, influenced by limited perfusion, low oxygen levels, mechanical stability, and inflammatory signals, steer the repair process toward endochondral progression.[15,36] As inflammation resolves, mesenchymal cells in the fracture gap preferentially adopt a chondrogenic fate, generating a stabilizing soft callus that later undergoes hypertrophic maturation and becomes permissive for neovascular entry (soft callus phase).[34,35] Vascular invasion is tightly coupled to cartilage turnover, enabling the emergence of osteogenesis and the formation of a mineralized hard callus, where woven bone replaces the cartilaginous template (hard callus phase). Subsequently, the woven bone remodels into a lamellar architecture, and marrow continuity is restored (bone remodeling phase).[33,34] Crucially, successful repair depends on correct sequencing and phase handovers—an error in timing can compromise later transitions even if individual pathways are strongly activated.[15,35]

From a developmental perspective, fracture repair highlights three design points: a transient callus forms first, cell phenotypes progress in order, and vascular invasion and remodeling follow hypertrophic maturation. The callus represents a poorly perfused, hypoxic tissue state that stabilizes the fracture gap early, then transitions to a strongly angiogenic hypertrophic program that licenses vascular invasion and ossification.[15] This transition requires coordinated matrix remodeling and vascular invasion, which supply oxygen, nutrients, and osteoprogenitors to replace the template with bone.[33,37] Seen this way, the key is not to maximize a single pathway, but to maintain the correct handover logic between phases that overlap in space and time.[15,33] This framing naturally foregrounds the fracture callus as a pivotal transient tissue state, which we discuss next as an instructive template for callus organoid engineering.[38]

2.3. Fracture callus as a transient, instructive tissue

Fracture callus is a transient, instructive tissue that serves as a functional template during endochondral fracture healing.[15,33] Histologically, the fracture callus contains spatially distinct yet coupled cartilaginous, mineralizing, and vascularizing regions, including transitional interfaces where cartilage is converted to bone.[15,33,39] Its functional importance lies in its ability to temporarily stabilize the defect environment while preserving the structural and biological conditions needed for subsequent regeneration.[40,41] Rather than acting as a passive cartilaginous intermediate, the callus becomes a biologically active tissue state that supports successful progression toward ossification.[37,41,42] In this view, the biological value of the callus lies not only in its composition, but in its capacity to support orderly transition from one regenerative state to the next. This transition competence is also conditioned by a dynamic osteoimmune milieu, in which macrophages play a crucial role in resolving inflammation, recruiting progenitor cells, remodeling the ECM, and facilitating the progression toward endochondral repair.[36,43]

When this progression is disrupted, callus tissue can stall, preventing the cartilage-to-bone transition from completing.[41,42] Clinically, delayed union and non-union reflect multifactorial failures that limit either the capacity to advance the program or the conditions required for transitions, and long-bone non-union remains a persistent challenge despite modern fixation and regenerative adjuncts.[44] Non-unions are often described as stalled phenotypes, such as hypertrophic and atrophic forms, highlighting that tissue accumulation does not guarantee productive transition.[44,45] For organoid design, this underscores that timing and phenotype control are first-order constraints, since failure can arise from loss of sequence as much as from the absence of cues.

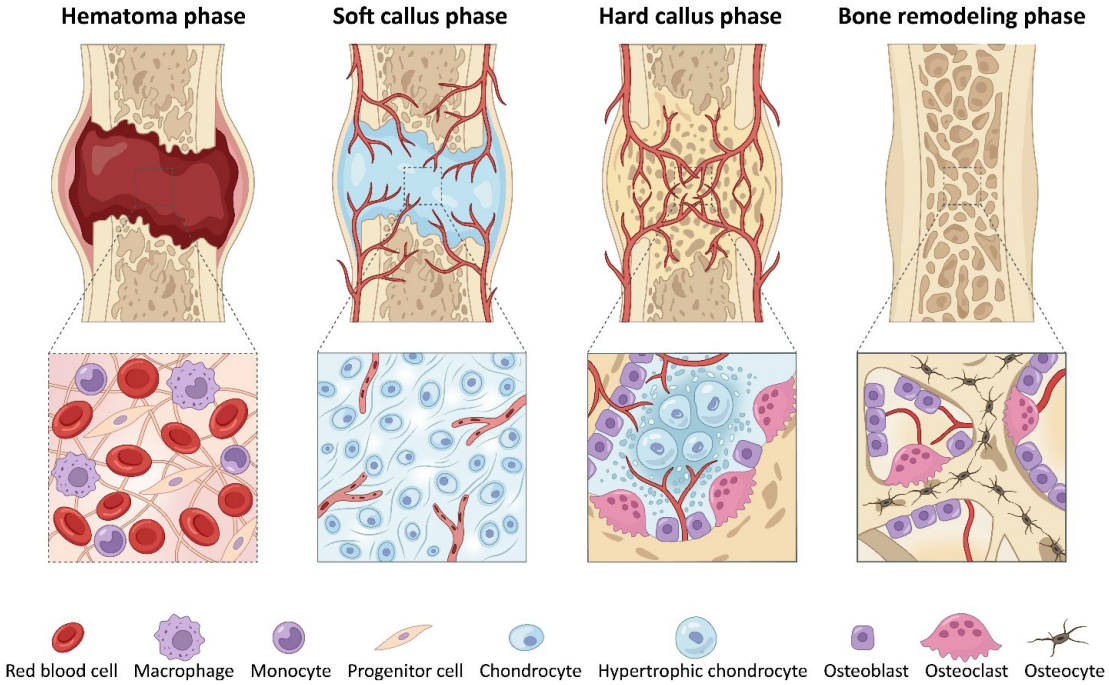

Figure 1. Schematic diagram of the endochondral ossification process with four phases. Image created by the authors using Adobe Illustrator (2026, Adobe Inc., USA).

2.4. Implications for developmental engineering

Fracture healing guides construct design by showing that repair requires stage-wise shifts in cell competence, ECM-dependent control of signal availability, and timely vascular and inflammatory input to drive cartilage-to-bone conversion.[14,39,46,47] Therefore, regenerative potency is best judged by whether a construct can progress through the repair sequence and complete the next transition, not by how closely it resembles an endpoint tissue. Following the same rationale, developmental fidelity should encompass not only lineage progression within the construct but also its capacity to effectively integrate with host osteoimmune regulation during post-implantation repair.[45,48] Callus organoids should be placed on this sequence at a defined maturation stage and evaluated after implantation by forward progression using transition-linked readouts rather than snapshot resemblance; recent studies support this by linking noninvasive secreted-factor signatures to *in vivo* outcomes.[18]

3. Callus organoids

3.1. Definitions of callus organoids

3.1.1. Organoid

Organoids are stem or progenitor cell-derived 3D tissue systems that self-organize *in vitro* and recapitulate key structural, cellular, and functional features of the corresponding organ or tissue.[49,50] A landmark example is the intestinal organoid system, in which single *Lgr5*$^+$ adult stem cells generated self-renewing crypt-villus-like epithelial structures *in vitro* that contain multiple differentiated intestinal cell types, establishing a canonical framework for organoids as self-organizing and lineage-diverse tissue models.[51] Cerebral organoids derived from human pluripotent stem cells were shown to form discrete brain-region-like domains and recapitulate key features of human cortical development, including progenitor-zone organization and outer radial glia.[52] Human induced pluripotent stem cell (iPSC)-derived liver bud systems, in contrast, self-organized with endothelial and mesenchymal cells into vascularized tissues that exhibited liver-specific functions after transplantation.[53] More recently, human heart-forming organoids have been shown to generate structured tissues containing myocardial, endocardial-like, vascular, and foregut endoderm components, thereby recapitulating aspects of early heart and foregut development.[54]

However, organoid-based systems are not limited to modeling stable mature tissue states. Developmental models such as gastruloids have shown that stem cell-derived 3D systems can be used to reconstruct staged developmental progression, including symmetry breaking, germ layer emergence, axial elongation, and lineage transitions, rather than only mature organ-like endpoints.[55] A related process-oriented logic is also seen in maternal-

fetal interface models, where organoids and assembloids are used to study implantation, decidualization, tissue invasion, and spatiotemporal coordination between maternal and fetal compartments, rather than to reproduce a static mature organ.[56] In the context of this review, the relevance of the organoid concept therefore lies not simply in 3D resemblance to native tissue, but in the ability of self-organizing cellular systems to capture biologically robust trajectories and transient/intermediate tissue states. This perspective provides the conceptual basis for the discussion of callus organoids below.

3.1.2. Callus organoid

Callus organoids are engineered 3D cellular constructs that recapitulate the key features of transient fracture callus. These constructs serve as developmental templates and can execute a semi-autonomous cartilage-to-bone transition post-implantation.[13,17,57] In this context, they are intended to model ECO for bone regeneration, rather than all possible mechanisms of bone repair. They are primarily characterized by their ability to follow a developmental sequence, typically progressing from condensation-like cell compaction to a chondrogenic state, then to a pre-hypertrophic or hypertrophic state, either established *in vitro* or initiated via *in situ* priming. This progression includes the development of maturation gradients and ultimately coupling with host vasculature to support ossification and remodeling *in vivo*, rather than by specific scales or fabrication methods.[12,58,59] In line with published studies, this progression can be interpreted in a stage-associated manner, with earlier callus-like states typically exhibiting chondrogenic markers, such as *SOX9* and *COL2A1*, whereas more advanced transition-competent states exhibit pre-hypertrophic or hypertrophic markers, such as *IHH*, *COL10A1*, and *BSP*.[17,18]

Notably, numerous relevant studies do not explicitly use the term "callus organoid," instead referring to cartilaginous microtissues, hypertrophic templates, or ossification center-like organoids, or related endochondral intermediate systems.[19,58,60] Figure 2 presents a tiered framework for the operational definition of callus organoids in the context of ECO-based bone regeneration. Within this framework, essential criteria include an engineered 3D living construct, a callus-like developmental trajectory, and

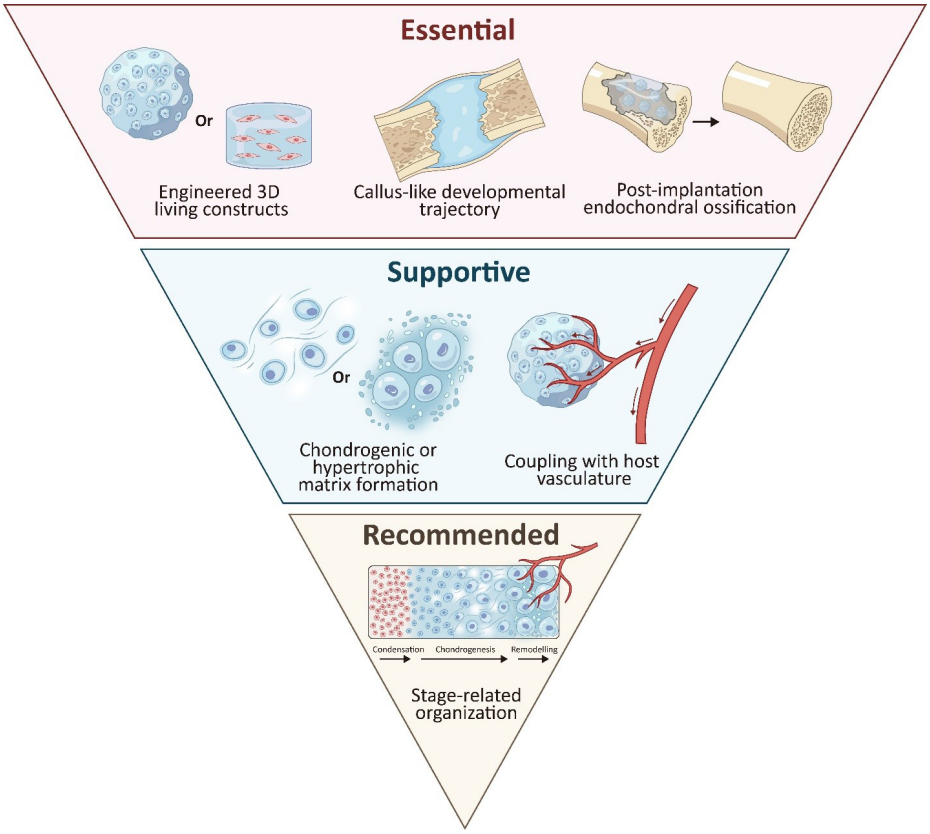

Figure 2. Proposed criteria for defining callus organoids. This schematic summarizes a tiered framework for the operational definition of callus organoids. This framework is intended to harmonize classification across studies with inconsistent terminology and to emphasize functional developmental identity over specific scale, fabrication method, or naming convention, while allowing stage-associated markers and *in vivo* transition readouts to be interpreted in context rather than as a fixed checklist. Image created by the authors using Adobe Illustrator.

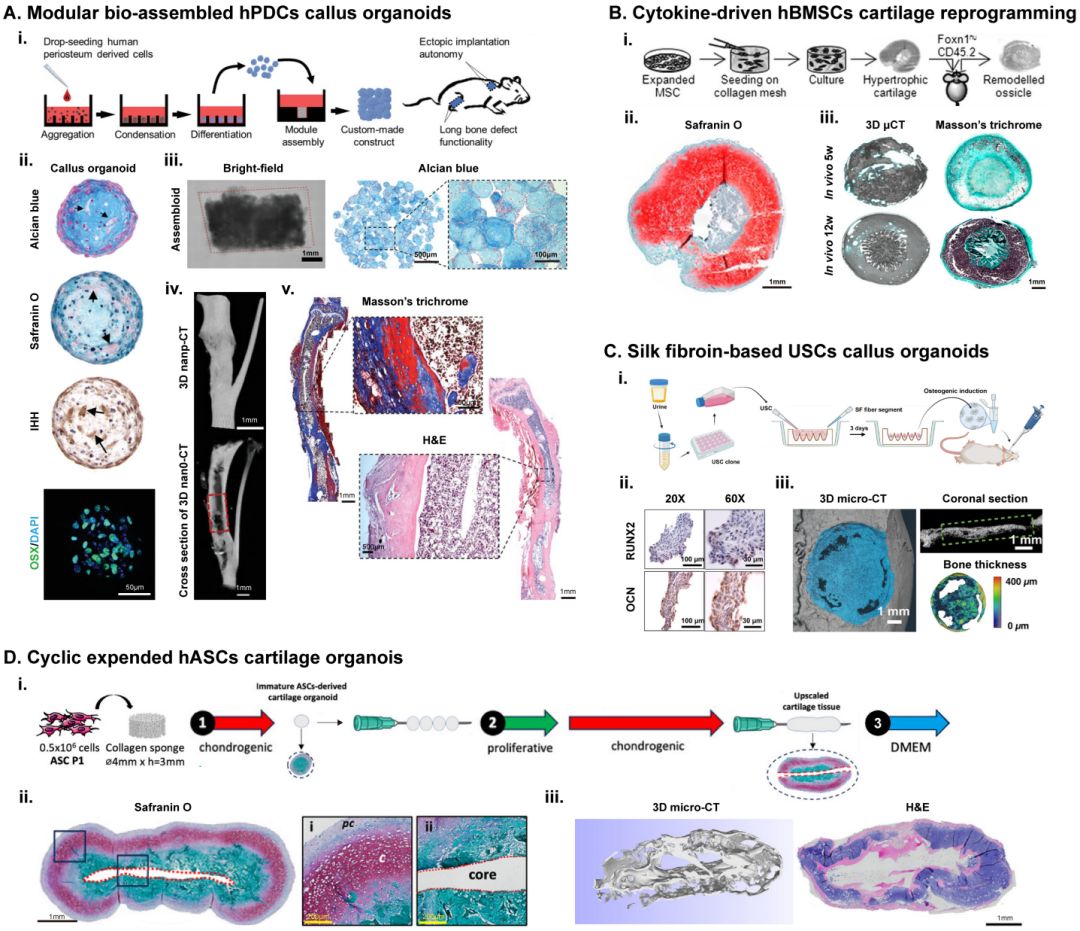

Figure 3. Strategies for developing callus organoids following endochondral ossification. Subpanels illustrate the engineering workflow for different callus organoid approaches. (A) Modular bioassembled human periosteum-derived progenitor cell (hPDC)-derived callus organoids. Reprinted from Ref.[58] (B) Cytokine-driven cartilage reprogramming to generate an endochondral ossification template from human bone marrow mesenchymal stromal/stem cells (hBMSCs). Reprinted from Ref.[17] (C) Callus organoids from urine-derived stem/progenitor cells (USCs) with silk fibroin. Reprinted with permission from Ref.[61] Copyright © 2025 Wiley. (D) Cyclic strategy for scaling cartilage organoids from human adipose-derived stromal/stem cells (hASCs). Reprinted from Ref.[62]

Table 1. Representative studies of callus organoids and callus-like endochondral constructs

Study	Cell source	*In vitro* identity	Culture system	Engineering strategy	Animal model
Scotti *et al.*[58]	hBMSC	D35 hypertrophic templates	Collagen sponge seeding	Scaffold-based seeding; generated cm-scale functional bone organ	Ectopic: subcutaneous (mouse)
Dang *et al.*[63]	hBMSC	D2 scaffold-free constructs	Scaffold-free microparticle-integrated high-density sheets	Microparticle-mediated assembly; *in situ* growth factor delivery	Orthotopic: 5 mm calvarial defect (rat)
McDermott *et al.*[64]	hBMSC	D2 mesenchymal condensations	Scaffold-free sheet/ tubular condensations	Self-assembly; programmable mechanical cue timing	Orthotopic: 8 mm femur defect (rat)

(*Cont'd...*)

Table 1. (*Continued*)

Study	Cell source	*In vitro* identity	Culture system	Engineering strategy	Animal model
Nilsson Hall *et al.*[17]	hPDC	D21 callus organoids	Scaffold-free spheroids	Modular self-assembly; ~6,000 organoids	Orthotopic: 5 mm tibia defect (mouse)
Xie *et al.*[57]	hBMSC	D21 osteo-callus organoids	DLP printed GelMA microspheres	Digital biomanufacturing; printed micro-niche building blocks	Orthotopic: 5 mm femur defect (rabbit)
Pitacco *et al.*[65]	hBMSC	D35 chondrogenic/ early hypertrophic templates	3D-bioprinted PCL/ fibrin as reinforced macro-construct	3D bioprinting of reinforced templates; central micro-channel for transport	Orthotopic: 5 mm femur defect (rat)
Peng *et al.*[66]	hPDC	D18 mesh-assembloids	Microtissues constrained within an MEW cage	Precision geometry; MEW physical constraint of callus organoids	Orthotopic: 5 mm tibia defect (mouse)
Ding *et al.*[61]	hUSC	D14 fiber-reinforced callus organoids	Fiber-reinforced organoid hybrid	Mass transport optimization; fiber-network reinforcement of organoids	Orthotopic: 5 mm calvarial defect (rat)
Xiong *et al.*[59]	hiPSC	Mature (6–8 weeks) growth plate organoids	Self-organizing organoid; complex spatiotemporal zonation	Lineage-directed self-organization; high-fidelity developmental control	Ectopic: renal capsule (mouse)
Pfister *et al.*[62]	hASC	Mature (5–12 weeks) Pa-TEC	Scaffold-free modules with fusion-promoting layers	Needle-guided assembly; cyclic priming and perichondrial-mediated fusion	Ectopic: subcutaneous (mouse)

Notes: Columns follow the review logic from cell source and *in vitro* identity to culture/engineering implementation and *in vivo* validation. This table highlights representative studies; additional callus organoid approaches are discussed in the main text. "*In vitro* identity" denotes the construct state upon implantation.
Abbreviations: 3D: Three-dimensional; DLP: Digital light processing; GelMA: Gelatin methacryloyl; hASC: Human adipose-derived stromal/stem cell; hBMSC: Human bone marrow-derived mesenchymal stromal/stem cell; hiPSC: Human induced pluripotent stem cell; hPDC: Human periosteum-derived progenitor cell; hUSC: Human urine-derived stem cell; MEW: Melt electrowriting; Pa-TEC: Phalanx tissue-engineered construct (as named in the cited study); PCL: Polycaprolactone.

competence for post-implantation ECO and remodeling; supportive features include chondrogenic or hypertrophic matrix formation and coupling with host vasculature and recommended features include stage-appropriate spatial organization within the construct, such as condensation-like compaction, chondrogenic matrix patterning, and the emergence of pre-hypertrophic or hypertrophic domains, reflecting a higher degree of developmental fidelity. Here, we use "callus organoids" as a functional umbrella term for these callus-like endochondral systems because inconsistent nomenclature has hindered retrieval and cross-study comparison across the field. Selected representative studies illustrating different engineering workflows for callus organoids following an ECO route are schematically summarized in Figure 3. To anchor this definition in practice, Table 1 maps representative callus organoid studies across cell sources, *in vitro* identity upon implantation, culture/engineering setups, and *in vivo* validation models.

3.2. Callus organoid as a developmental engineering construct: Distinctions from classical bone tissue engineering and bone organoids

3.2.1. Classical bone tissue engineering versus callus organoids: Top-down vs. bottom-up

Classical BTE is an alternative regenerative strategy that involves seeding a patient's own stem or progenitor cells into designed scaffold matrices to restore damaged bone function.[67] Bone is viewed as a complex, mechanically responsive tissue sustained by interactions among cells, mechanical forces, and a constantly remodeling matrix.[67] Classical BTE integrates the four essential elements of

the "tissue engineering quad": biomaterial scaffolds, regenerative cells, morphogenetic signals (e.g., growth factors), and advanced fabrication technologies.[68]

Accordingly, constructs are developed through a top-down engineering workflow, often using 3D printing methods, such as fused deposition modeling or selective laser sintering, to achieve defect-matched geometry and predictable mechanical performance.[68,69] Beyond construct geometry and mechanics, scaffold-guided BTE has also explored material platforms and local delivery strategies designed to support vascularization, osteoconduction, and biological instruction.[70–74] However, engineering large-volume grafts remains constrained by nutrient diffusion limits and delayed vascular ingrowth, which can compromise cell survival and spatially uniform maturation.[75,76] To address vascularization, prefabrication strategies use the body as a living bioreactor, for example, by endocultivating constructs within a vascularized muscle bed (e.g., latissimus dorsi) or by applying microsurgically created arteriovenous loops to induce axial vascularization before transfer.[76–78]

Taken together, classical BTE embodies a top-down, construct-centric philosophy: biological function is pursued through manufacturable control over geometry, mechanics, and exogenous instructive cues. Callus organoids, in contrast, follow a developmental engineering logic that is fundamentally bottom-up and process-driven. Rather than directly fabricating "finished" bone, they leverage self-organization and staged lineage progression to recreate the regenerative microenvironment of fracture healing and to guide subsequent tissue conversion *in vivo*.

3.2.2. Bone organoids versus callus organoids: Endpoints vs. transitions

Bone organoids are 3D self-organizing constructs that generate bone-like tissue features *in vitro*.[9] Most models follow direct osteogenic differentiation with matrix mineralization, resembling an intramembranous ossification route.[9,79] Bone organoids have evolved from scaffold-free cellular condensations to engineered systems that provide osteoid- and bone-mimetic cues, such as mineralized or osteoconductive biomaterials and printed architectures.[80–83] Maturation is commonly assessed by osteocyte embedding and osteocyte-associated readouts (e.g., sclerostin), sometimes accompanied by lacuno-canalicular-like features.[84,85]

Despite significant advancements, most bone organoid systems still fail to adequately replicate the complex physiology of mature bone. This includes challenges in achieving long-term lamellar stability, sustained remodeling, and integrating bone marrow-associated components.[9,86] Therefore, bone organoids primarily aim toward a durable, homeostatic bone-like endpoint *in vitro*. In contrast, callus organoids are engineered, within a developmental engineering framework, as a transient intermediate programmed for post-implant cartilage-to-bone transition, rather than long-term *in vitro* maturation toward a stable bone endpoint.

3.3. Historical development and proof-of-concept validation

While "callus organoids" as a term dates to Nilsson Hall *et al.*[17], one of the earliest studies of the endochondral potential of 3D cultures was provided by Zimmermann *et al.*[87] Their study showed that self-organized mouse embryonic limb bud cells could form "cartilage organoids" capable of mineralizing *in vitro* via phosphate supplementation. Subsequently, Johnstone *et al.*[88] developed a robust rabbit mesenchymal stem cell (MSC) pellet culture system for chondrogenesis, laying a technical foundation for scaffold-free cartilage templates.

Using human MSC-derived cartilage templates, hypertrophic maturation was shown to be critical for reliable *in vivo* bone formation.[21] Consistently, chondrogenic priming, rather than osteogenic priming, was essential for consistent bone and marrow development using human and rat MSCs, reinforcing cartilage priming (and subsequent hypertrophic progression) as a more predictive route than osteogenic priming alone.[89] Collectively, these studies established ECO as a biologically robust strategy for bone regeneration. Hypertrophic cartilage-based approaches were used to generate large, vascularized bone tissues *in vivo* through integration with supporting scaffolds and inflammatory stimulation.[58] Dang *et al.*[63] introduced a modular, scaffold-free system utilizing self-condensed MSC sheets embedded with morphogen-releasing microparticles, which effectively bridged critical defects through *in vivo* cartilage formation and remodeling. Importantly, engineered human mesenchymal condensations paired with mechanical cues showed that the timing of loading is a decisive design variable for bridging outcomes, reinforcing maturation control as a core engineering lever immediately prior to the "callus organoid" framework.[64]

The progression of research culminated in the work of Nilsson Hall *et al.*[17], who introduced the term "callus organoids" to refer to microspheroids derived from hPDCs. These organoids exhibit chondrogenic and hypertrophic differentiation *in vitro*, autonomously progressing into osteogenic units that self-assemble into larger structures. Upon implantation into critical-sized mouse tibial defects, they promoted defect bridging and bone regeneration. In summary, a decade of developmental engineering research

has substantiated the capacity of callus organoids to effectively form bone ectopically and to heal critical defects, thereby validating this concept across multiple laboratories.

4. Design principles linking fracture biology to engineering callus organoids

This section translates fracture-healing biology into practical engineering decision points that determine whether a construct behaves as a callus organoid rather than generic cartilage or bone. We focus on what can be controlled experimentally, namely the cell starting state, the ordered sequence of state transitions, and the maturation or implantation time point, because these variables shape robustness, comparability, and translatability.[14,46]

4.1. Cell-intrinsic requirements

A suitable callus-organoid cell source should prioritize endochondral competence over osteogenic potential. It should progress through stable cartilage intermediate formation, mature into hypertrophy, and transition to ossification without premature mineralization or incomplete cartilage development.[17,46,90] In practice, periosteum- and marrow-derived progenitors are preferred when accessible due to their reliable cartilage-to-bone progression and adaptability to changes in donor biology.[10,17,18] For translation, cell choice should be aligned with manufacturability and potency-linked quality attributes. For example, non-destructive soluble glycosaminoglycan (GAG) release has been proposed as a release criterion to flag resorption-prone adipose-derived batches, highlighting the importance of standardized reporting to enable cross-study comparability.[62,91]

4.2. Programming developmental trajectories

Programming a callus organoid involves initiating and controlling a time-ordered sequence of cues to guide tissue development along a defined developmental trajectory, rather than driving the construct toward a single differentiation endpoint.[46] Developmental engineering assesses constructs by fidelity to the intended trajectory, rather than by individual markers or bulk analyses that overlook spatial and functional heterogeneity.[14,92] In practical terms, this trajectory should be resolved into a limited number of critical transition stages that are directly relevant to callus organoid design: establishment of a stable chondrogenic callus-like state, progression to a pre-hypertrophic/hypertrophic transition-competent state, and subsequent entry into a vascular-permissive ossification handover stage.

A common failure mode is either arrest at an immature chondrogenic stage or premature drift toward mineralization before a functional pre-hypertrophic/hypertrophic transition window is reached, thereby compromising vascular integration, remodeling, and reliable defect bridging.[20,93] Microtissue models make this risk explicit: brief or mismatched growth-factor exposure can shift microtissues toward divergent chondrogenic versus hypertrophic responses, highlighting how "stage errors" arise when process inputs are treated as simple factor stacking.[94] Conversely, insufficient or poorly timed priming can leave constructs unable to trigger robust endochondral progression after implantation, underscoring that the pre-hypertrophic/hypertrophic window is not merely a later differentiation stage, but the critical transition state at which the construct remains callus-like while becoming permissive to vascular-coupled endochondral progression.[46,90] Stage-associated markers, such as persistence of chondrogenic markers (e.g., *SOX9* and *COL2A1*) together with emergence of pre-hypertrophic/hypertrophic markers (e.g., *IHH* and *COL10A1*), can help position constructs within this window, although marker expression alone does not prove successful phase handover.[17,18] Therefore, keeping the trajectory on track should be treated as a core design requirement rather than a late-stage optimization.

Since the "product" is a changing developmental stage, studies can only be compared if they clearly state which transition stage the organoids represent, their maturity, and whether stage assignment is supported by stage-associated markers, matrix features, or functional transition-linked readouts. Reviews of MSC translation repeatedly point out that inconsistent reporting of critical parameters undermines reproducibility and cross-study benchmarking.[91] For callus organoids, stage-aware reporting is also a manufacturing issue: noninvasive morphometrics and process-linked quality attributes can help determine whether batches remain within the intended chondrogenic or transition-competent pre-hypertrophic/hypertrophic window, enabling meaningful comparisons and, later, scale-up.[14,19] Clear stage reporting also prepares the ground for later discussions on manufacturing consistency and scale-up.

4.3. Temporal control of organoid maturation

Timing is a critical design parameter because the same construct can be a different biological "product" at different stages of maturation (e.g., pre-hypertrophic, hypertrophy-primed, or mineralizing). These stages differ in matrix state and in how they couple to host vascular invasion and remodeling, so "when to implant" is a design decision rather than a procedural detail.[46,92] In the context of designing implantation-oriented callus organoids, a critical temporal consideration is determining whether the construct has reached a transition-competent pre-hypertrophic or early hypertrophic stage, without advancing to the point at which its transient callus-like characteristics are compromised.[17,21]

One practical implication is that timing could be paired with non-destructive release logic: pre-implant GAG release in spent medium has been proposed as a release criterion that predicts *in vivo* ossification performance, and secretome-level biomarker panels have been framed as noninvasive potency indicators for callus organoid manufacturing.[18,62] Thus, maturation time should be stated and justified as rigorously as the cell source or biomaterial choice.

Timing control becomes increasingly difficult as constructs increase in size or complexity, because diffusion limits, the window for a vessel-permissive stage, and the practical constraints of assembly and handling must be coordinated. Size and packing density impose transport constraints that can shift maturation trajectories in spheroid and organoid systems.[95,96] In this context, vascularization is also a temporal challenge: constructs must reach a stage permissive to vessel invasion without exceeding the diffusion support available before perfusion is established.[97] In most laboratory settings, these challenges are still addressed mainly through empirical control of construct size, culture duration, and implantation stage, rather than through real-time process monitoring. More advanced image-based monitoring approaches, including automated analysis, may help improve staging consistency and quality control, but their broader implementation remains technically demanding and typically requires dedicated platforms, standardized imaging conditions, and robust validation across batches.[19,98,99] These practical constraints should therefore be taken into account when discussing temporal control in callus organoid engineering and when evaluating subsequent construction and biofabrication strategies.

5. Strategies for callus organoid construction

5.1. Cell sources

5.1.1. Periosteum-derived progenitor cells

Periosteum-derived progenitor cells are a heterogeneous osteochondral progenitor population residing in the periosteum, a tissue niche that is rapidly activated after fracture.[100,101] In long-bone repair, periosteal progenitors are consistently positioned among the key local contributors to the cartilage and bone that form within the fracture callus, which makes periosteum a biologically aligned starting point for engineering callus-like tissues for bone defect repair.[100,102,103] Recent periosteal progenitor atlases further support a staged injury response that transitions toward chondrogenic and osteogenic trajectories, conceptually matching the endochondral logic that underpins callus organoid design.[104–106]

For translational callus organoid engineering, hPDCs are attractive because they are tissue-relevant for callus formation and can be guided toward a cartilage-to-bone sequence rather than relying only on direct osteogenesis.[107] In a translationally oriented workflow, serum-free preconditioning combined with microaggregate assembly and bone morphogenetic protein (BMP)-2 priming enabled human periosteal cells to support bridging of a critical-sized murine long-bone defect, consistent with an endochondral-like repair trajectory.[108] Importantly, moving toward robust manufacturing will require potency-linked quality attributes. Single-cell-informed enrichment of *BMPR2*$^{+}$ hPDCs provides one example of a marker-guided strategy to enrich a more osteochondrogenic subset and link *in vitro* characterization to *in vivo* defect-healing performance.[109]

The clearest callus organoid proof-of-concept for defect repair is the developmental-engineering route, where hPDC microspheroids are matured into "callus organoids" exhibiting distinct late pre-hypertrophic characteristics before being bioassembled into scalable, scaffold-free implants.[17] In this paradigm, day-21 modules were operationally defined as callus organoids, and ~6,000 units were fused into a construct sized for a murine critical-sized tibial defect; longitudinal imaging showed mineralization by two weeks, functional bridging around four weeks, and progressive corticalization by eight weeks, ultimately yielding a regenerated structure approaching native tibial morphology.[17]

Moving forward, the primary bottleneck remains scaling these organoids into large-scale implants without compromising endochondral kinetics or delaying vascular integration. Recent findings suggest that, under typical *in vitro* culture conditions, diffusion constraints impose an effective length scale on the order of 1 mm for maintaining viability and relatively uniform differentiation.[110] A translational route involves pairing organoid maturation with automated biofabrication, such as image-guided robotic assembly or bioprinting constructs with preformed channels and interconnected porosity. Such architectures can accelerate vascular ingress and perfusion while maintaining the staged cartilage-to-bone sequence that underpins regenerative potency.[100,110]

5.1.2. Bone marrow-derived progenitor cells

Bone marrow-derived progenitor cells are most commonly implemented as culture-expanded bone marrow mesenchymal stromal/stem cells (BMSCs), a clinically familiar progenitor pool that can be manufactured as 3D condensations or microtissues and then guided into defined endochondral "states." A central advantage of callus organoid-inspired bone defect repair is this state controllability: hypertrophic cartilage-biased BMSC tissues can be generated *in vitro* and later remodel into bone *in*

vivo, offering a practical blueprint for staged fabrication rather than relying on direct osteogenesis alone.[21,89] This programmability has enabled defect-oriented, callus-analog living constructs, from engineered mesenchymal condensations to explicitly termed "osteo-callus organoids." This positioned marrow progenitors as a pragmatic cell source for callus-inspired organoid manufacturing.[57,64,111] Accordingly, BMSCs are best viewed here as a scalable progenitor platform for producing callus organoids where the engineered state of the construct largely defines reparative function.

Representative studies position numerous BMSC-based endochondral living implants as functional callus-organoid analogs for bone defect repair: they are 3D, matrix-producing cellular assemblies that traverse a cartilage-like intermediate and remodel within the defect niche. Developmental-engineering work established hypertrophic cartilage templates engineered from adult human MSCs that subsequently remodel into bone *in vivo*, and chondrogenically primed MSC constructs likewise formed bone via ECO after implantation.[21,89] Defect-oriented mesenchymal condensations refined this concept into a deliverable "callus module," where morphogen presentation and fixation-enabled loading tuned cartilage persistence, vascularization, and bridging.[64,111] At the explicit organoid end, BMSC-laden micro-units were assembled and staged into "osteo-callus organoids" that accelerated large-defect healing.[57] In the Martin ecosystem, engineered hypertrophic cartilage matrices were also devitalized via an inducible apoptosis genetic device while retaining osteoinductivity, highlighting how engineered (non-ordinary) MSC sources can be used to standardize callus-like graft manufacturing.[112]

Cell-source-specific limitations remain important in a comparative "cell sources" section. Marrow procurement requires aspiration, with recognized pain/morbidity and low MSC frequency, whereas adipose tissue can provide abundant stromal cells via minimally invasive harvesting.[113] In parallel, periosteal tissue can often be collected from sites exposed or discarded during orthopedic procedures, potentially simplifying access relative to separate marrow aspiration.[114] Biologically, BMSC endochondral potency is strongly donor- and expansion-state dependent; in an orthotopic non-union model, outcomes were explicitly donor-dependent even under standardized chondrogenic induction.[115] These constraints motivate tighter quality-control criteria linked to hypertrophic trajectory and, where feasible, more standardized manufacturing inputs when BMSCs are used to build callus organoids.

5.1.3. Induced pluripotent stem cells

Induced pluripotent stem cells are a useful cell source for callus organoid construction because they can be expanded at scale and, more importantly, guided through defined developmental steps before 3D self-assembly. This allows the starting state to be set more precisely than with heterogeneous adult stromal isolates. In practice, pluripotent cells can be directed into skeletal progenitor trajectories, including *SOX9*$^+$ sclerotome-related progenitors, and can also be biased toward distinct cartilage fates (e.g., stable articular-like vs. hypertrophy-prone programs) depending on the signaling stimulation.[59,116] For callus organoids, this matters because the goal is not terminal bone or stable articular cartilage, but a transient cartilage template that can progress through condensation, enter a controlled hypertrophic window, and then support vascular-coupled remodeling *in vivo*.

Functional support for this concept has been provided in studies showing that iPSC-derived chondrocytes can self-assemble into scaffold-free cartilaginous organoids and promote bridging of critical-sized long-bone defects.[117] A recent study has refined sclerotome-based induction to generate near-uniform *SOX9*$^+$ progenitors at a pre-condensation stage, which then undergo condensation and progress through key steps of ECO, reinforcing the idea that iPSCs can be used to program a callus-like intermediate rather than to directly make bone *in vitro*.[59]

Moreover, a practical advantage of iPSC systems is that process control can be built into manufacturing. A *COL2A1*-green fluorescent protein knock-in human iPSC line has been used to enrich chondroprogenitors and improve consistency of chondrogenic output before 3D assembly.[118] In addition, real-time, non-destructive tracking of the chondrogenesis-to-hypertrophy transition has been demonstrated with *COL2A1*/*COL10A1* double-reporter pluripotent systems in a cartilaginous organoid platform (reported in human expanded pluripotent stem cells), illustrating how maturation windows can be monitored non-destructively without consuming the construct, thereby supporting the generation of endochondral-competent organoids, effectively aligning the starting cellular state with the dynamic requirements of the fracture-healing blueprint.[119]

5.1.4. Alternative and non-canonical sources

Adipose-derived stromal/stem cells (ASCs) and stromal vascular fraction are accessible, high-yield sources for callus-inspired living constructs.[120] Their advantage is scalability; maintaining niche cues may counteract the loss of *in vivo* osteogenic potency after monolayer expansion.[121] In developmental-engineering paradigms, ASCs can be primed into (pre)hypertrophic cartilage templates as callus organoids that remodel into ossicles via ECO.[122] Fractionated-fat "Adiscaf" constructs simplify manufacturing and enhance ECO-driven bone/marrow formation, and dispersing calcium-phosphate granules

improves inter-donor robustness.[123,124] Cyclic "start–stop–rewind" protocols further enable organoid fusion into scalable, shape-defined hypertrophic grafts.[62] Compared to skeletal progenitors, adipose-derived cells typically show a weaker baseline skeletogenic bias and are more sensitive to expansion history, thereby requiring stricter chondrogenic priming and stage control for reproducible ECO.[121,125,126]

Urine-derived stem/progenitor cells (USCs) are clonogenic, MSC-like cells that can be isolated from voided urine and expanded *in vitro*.[127] Their appeal for defect-oriented callus organoids lies in noninvasive, repeatable autologous sourcing with strong proliferative capacity, thereby bypassing painful marrow harvest and enabling patient-specific manufacturing.[128] Early BTE work showed that USCs remained viable and osteogenically differentiated within β-tricalcium phosphate and enhanced bridging of a 6 mm critical femoral defect in rats.[129] Critically, Ding *et al.*[61] provided a canonical "USC-derived callus organoid" example: USCs self-organized within a cell-aligned silk fibroin fiber network to form porous callus organoids within two weeks; upon implantation into 5 mm cranial defects, these organoids accelerated repair with improved micro-computed tomography and histological outcomes at eight weeks. Key barriers remain low/variable yield, donor heterogeneity, and the need for standardized collection and Good Manufacturing Practice-compatible workflows.[128]

Skeletal muscle contains abundant progenitor cells, including satellite cells and muscle-derived stem cells (MDSCs), which are readily accessible via biopsy.[130] Notably, MDSCs can differentiate into osteogenic and chondrogenic lineages and contribute to bone and cartilage repair in response to appropriate stimuli.[130] In fracture contexts, cells harvested from muscle adjacent to fracture sites differentiate into osteoblasts and form bone nodules *in vitro*, with potency equivalent to bone marrow stromal cells.[131] Lineage-tracing studies confirm muscle's participation in callus formation: for example, myogenic differentiation 1-lineage muscle cells accounted for ~50% of the callus in open tibial fractures and gave rise to cartilage and bone.[132] Similarly, paired-related homeobox 1 (Prx1)-derived mesenchymal progenitors in skeletal muscle directly contribute to callus cartilage and bone.[133] However, a callus organoid specifically derived from MDSCs has not yet been reported.

5.2. Biochemical regulation

Biochemical regulation in callus organoid systems is primarily designed to guide cells through successive developmental states that resemble fracture repair and skeletal development, rather than to impose a fixed terminal phenotype.[13,21] This concept is central to developmental engineering, which aims to harness endogenous morphogenetic programs by reproducing the temporal sequence of developmental cues rather than maximizing lineage-specific differentiation *in vitro*.[16,134–136] Endochondral strategies therefore focus on recapitulating mesenchymal condensation, chondrogenesis, hypertrophy, vascular invasion, and ossification, mirroring the natural fracture callus cascade.[137,138] In this context, biochemical cues are applied to steer transitions between developmental states rather than to lock cells into osteoblastic phenotypes prior to implantation.[21] This strategy exploits the intrinsic ability of cartilage templates to recruit host vasculature and remodel into bone *in vivo*, reducing the need for extensive osteogenic induction *in vitro*.[14,16,92,136]

5.2.1. Growth factor-guided lineage progression

Growth factor-guided lineage progression typically begins with chondrogenic priming using transforming growth factor (TGF)-β family members to induce cartilage matrix formation in a 3D culture system as pellets, microtissues, or organoids.[88,139] This early chondrogenic phase establishes a cartilage template that resembles the soft callus formed during fracture healing.[137] Progression toward hypertrophy and endochondral competence is achieved by withdrawing chondrogenic cues and introducing pro-hypertrophic or osteogenic signals such as BMPs, thyroid hormone, or mineralization-permissive conditions.[11,89,111,140–145] Thus, two principal strategy classes have emerged for temporally controlling biochemical cues in 3D endochondral-like BTE systems: (i) material encoded morphogen programs and (ii) discrete stagewise media schedules.

In the first class, scaffold-free human MSC condensations, rings, or tubes are loaded with microparticles that deliver TGF-β1 rapidly and BMP-2 in a delayed or sustained fashion, thereby hard-wiring an early chondrogenic and later osteogenic phase into the construct without relying on repeated medium exchanges.[63,111,141,142,146] In the second class, temporal control is imposed via explicit media switches, typically a chondrogenic phase (TGF-β family) followed by a hypertrophic/osteogenic phase (TGF-β withdrawal, BMP/Wingless-related integration site [Wnt]/β-glycerophosphate [BGP]/triiodothyronine addition), and in more advanced systems a third vascular phase (human umbilical vein endothelial cell [HUVEC] co-culture or vasculogenic matrices), with priming duration and switching time treated as design variables. Canonical developmental engineering protocols chondrogenically prime MSCs in 3D culture to generate cartilage templates that undergo vascularized bone and marrow formation after implantation[21,58], while comparative studies show that chondrogenic priming (endochondral route) yields more robust bone repair and vascularization than direct osteogenic priming (intramembranous route) in critical-sized bone defects.[12,21,58,89,147] Systematic variation of

chondrogenic priming time in pellets and constructs reveals non-monotonic dependencies, with intermediate priming windows maximizing subsequent hypertrophy and mineralization *in vitro* and ECO bone formation *in vivo*, and "brief priming" micropellet systems extending these insights to scalable microtissues.[148–150] Multi-phase protocols that add a defined hypertrophic/mineralizing stage (e.g., chondrogenic–hypertrophic cartilage–partially mineralized template) and then a vascular priming stage (e.g., HUVEC co-culture in vasculogenic hydrogels) further refine this temporal logic, producing prevascularized hypertrophic cartilage grafts that show enhanced vascularization and bone formation upon implantation.[11,140,143–145,150,151] Together, these studies support the interpretation that growth factors function as regulators of lineage progression and state transitions, rather than as direct inducers of terminal osteoblast differentiation.

5.2.2. Temporal modulation rather than biochemical saturation

A defining characteristic of callus organoid strategies is the temporal modulation of biochemical cues instead of continuous or saturating growth factor exposure.[14] Across advanced ECO models, a central design principle is the temporal introduction and withdrawal of specific growth factors to choreograph late stages of the cartilage-to-bone transition, particularly mineralization and angiogenesis. After an initial TGF-β-driven chondrogenic phase, mineralization is typically induced by delayed addition of osteogenic cues such as BGP and/or increased BMP signaling, often coincident with reduction or withdrawal of TGF-β, to shift *SMAD* signaling from a *SOX9*-dominated chondrogenic program toward Runt-related transcription factor 2-driven hypertrophy and matrix mineralization.[93] Ji *et al.*[93] showed that introducing BGP only after two weeks of TGF-β chondrogenic priming, and withdrawing TGF-β at day 14, generates a mineralized cartilage state but simultaneously reduces VEGF-A production and impairs endothelial migration and tube formation, indicating that mineralization introduced too early or too aggressively can be anti-angiogenic and that pro-angiogenic windows are tightly linked to the timing of hypertrophy and mineral deposition.

Similar logic underpins *in vivo*-oriented constructs in which TGF-β is restricted to an early phase, and BMP-2 is delivered in a more sustained, delayed manner from internal microparticles, so that endochondral commitment and remodeling are driven by a built-in temporal sequence of morphogen exposure rather than constant co-stimulation.[111,146,152] In parallel, vascularization is rarely promoted from the outset; instead, angiogenic cues are introduced only after a chondrogenic (and often early hypertrophic) state is established, for example, by switching from chondrogenic to endothelial growth media and adding HUVECs as a second, vascular priming phase, or by encapsulating hypertrophic microtissues in vasculogenic fibrin hydrogels at a defined time point.[143,145,153,154] Collectively, these studies demonstrate that TGF-β withdrawal, the introduction of BMP/BGP, and other osteogenic factors, and angiogenic stimulation are independent, tunable variables that strongly influence whether engineered cartilage progresses to vascularized bone tissue via a faithful endochondral sequence.

5.3. In vitro culture paradigms

The *in vitro* cultivation paradigm is a decisive process variable that steers the developmental trajectory of callus organoids, defines operable maturation windows, and helps manage donor-to-donor and batch-to-batch variability.[14,155] These platforms are not merely containers. Instead, they function as process-defined niches that prioritize one of three aims: promoting scaffold-free self-organization through high-density condensation, guiding morphogenesis with tunable material constraints, or improving transport and synchronization via engineering-assisted dynamic culture.[14,19]

5.3.1. Scaffold-free self-organizing culture

Scaffold-free approaches prioritize cellular self-organization, relying on endogenous ECM deposition to emulate the mesenchymal condensation that initiates natural ECO.[57,154] Conceptually, this aggregation is often mediated by cell-adhesion molecules such as N-cadherin, which facilitates the initial cell–cell binding necessary to trigger chondrogenic programs.[10,156] In practice, condensations are commonly generated by forced aggregation in microwells, U-bottom low-adhesion plates, or hanging-drop formats, producing size-controlled microtissues. These microtissues are then conditioned in suspension using staged chondrogenic-to-(pre)hypertrophic cues. Across hPDC and BMSC-based systems, this program supports progression from a *SOX9*$^+$ chondrogenic state toward a *COL10A1*$^+$/*IHH*$^+$ (pre) hypertrophic phenotype.[17,21]

This staged maturation provides a practical basis to describe late-stage microtissues as callus organoids primed for cartilage-to-bone conversion upon implantation.[17] Beyond developmental fidelity, scaffold-free constructs function as modular "living units" uniquely suited to validate tissue autonomy—the threshold at which a tissue intermediate can independently drive its own maturation and remodeling after the withdrawal of exogenous cues.[58,117] This paradigm has been successfully expanded to include non-canonical cell sources, such as USCs, which self-organize into porous callus-like constructs, and iPSC-derived sclerotome progenitors.[61,157,158] At the structural level, these condensations can be manipulated into defect-

relevant geometries, such as macro-scale tubular structures that mimic long-bone diaphyses and facilitate host vascular invasion.[64,146]

However, the lack of external physical support increases susceptibility to geometric drift and batch heterogeneity during prolonged maturation.[19] Moreover, as microtissues expand or merge into larger assembloids, diffusion limitations impact metabolic uniformity.[14,110] Exceeding this critical dimension significantly increases the risk of core hypoxia, unintended cell loss, and phenotypic deviation from the target transition.[87,110] These limitations necessitate the integration of material-based reinforcements or dynamic engineering systems to enable the production of large-scale, functional bone grafts.

5.3.2. Hydrogel-supported culture

Hydrogel-supported paradigms utilize tunable microenvironments to regulate organoid/spheroid density, preserve construct geometry, and facilitate the programmed bioassembly of organoids into anatomical shapes.[159,160] Photocrosslinkable matrices such as GelMA[161] and HAMA[160], alongside natural collagen[162] or fibrin gels[163], or alginate-based hydrogels[164,165] provide structural frameworks that balance cell-mediated remodeling with mechanical stability.[83,166]

Architectural modifications further refine these systems; for instance, incorporating porous silk fibroin fiber networks has been shown to optimize mass transport and reduce oxidative stress, thereby sustaining high cell viability in high-density organoid assemblies.[61] Moreover, hydrogel platforms enable the spatial patterning of microtissue units through extrusion-based bioprinting or self-healing bioinks, providing a practical route for engineering complex, zonally organized callus templates.[159,167]

These benefits come with a trade-off: matrix stability must be balanced against biological remodeling. Overly dense or weakly degradable hydrogels can act as physical barriers to host neovascularization (*CD31*$^+$) and delay cartilage-to-bone conversion.[14,160] From a translational perspective, material choice can also introduce regulatory friction because only a subset of formulations has clear clinical precedents for implantation. This motivates the use of clinically established materials or strategies in which hydrogels serve as temporary or sacrificial process aids rather than as permanent graft components.[19]

5.3.3. Dynamic culture and in-process monitoring

Engineering-assisted dynamic systems are typically incorporated as constructs scale up or as throughput requirements increase. Their primary roles are to alleviate mass-transport bottlenecks and promote batch synchronization, thereby reducing within-batch dispersion in developmental state.[14,19] Representative formats include orbital shaking, spinner flasks, rotating-wall vessels, controlled stirred-tank bioreactors, including stirred microbioreactors, in which an operating window balances suspension with intermittent shear exposure, as well as perfusion-based systems for larger or more densely assembled grafts.[155,168] By introducing controlled convection, these platforms can attenuate size-dependent nutrient and oxygen gradients and reduce cellular stress, both of which are common contributors to phenotypic drift during scale-up.[110,155]

Automation further enables noninvasive, in-process monitoring and supports a quality-by-design mindset. Integrated robotic workflows coupled with brightfield imaging are often combined with machine learning-assisted analysis. They enable real-time extraction of morphometric attributes, such as fusion dynamics and shape descriptors (e.g., solidity), to track whether organoid populations remain within a mineralization-permissive window.[19,110] These digital readouts can be complemented by spent-medium analytics, such as metabolite panels, to provide potency-linked process information.[18,168] For example, soluble GAG accumulation in the supernatant has been used as a non-destructive surrogate of chondrogenic matrix output, and in a defined platform, a threshold (e.g., >150 μg/week) was proposed as a release criterion associated with improved prediction of *in vivo* bone formation.[18,62] Importantly, such thresholds are best interpreted as platform- and workflow-dependent rather than universal.

Beyond routine manufacturing control, organoid-on-a-chip systems extend process understanding by enabling real-time modeling of early vascular dynamics under defined microfluidic conditions. These platforms are useful for interrogating early interactions between endothelial cells and mesenchymal condensations and for validating "handover" windows that precede robust vascular invasion.[157,169,170] Collectively, dynamic culture combined with in-process monitoring shifts bioreactors from simple production enablers toward decision-support frameworks that strengthen batch comparability across variable donors and increase confidence in translational workflows.[14,19]

Together, these considerations indicate that callus organoid construction depends on the coordinated selection of cell sources, temporally controlled biochemical cues, and appropriate *in vitro* culture paradigms, as summarized in Figure 4.

6. Engineering strategies for bone defect repair

6.1. Self-assembly and assembloid-based approach

Current strategies in BTE are increasingly shifting toward

bottom-up developmental engineering, in which living cell modules are first formed and then combined into a graft, rather than using a large scaffold as the main template.[14] In this setting, periosteum-derived progenitors and marrow-derived stromal cells are guided to form a cartilage intermediate similar to the fracture soft callus, which can later remodel into vascularized bone through ECO.[58] Several self-assembly routes have supported this idea. Scaffold-free, high-density MSC condensations with staged morphogen cues can follow a cartilage-to-bone sequence.[63] Studies also show that condensation shape (for example, tubes versus sheets) can affect morphogen exposure and regeneration outcomes, making assembly design an important parameter.[146] Building on this foundation, organoid-level modularity has enabled more scalable assembly: hPDC-derived callus organoids can fuse into larger bioassemblies and were reported to bridge murine critical-sized long-bone defects while forming mineralized tissue with a marrow compartment.[17] In addition, assembloid strategies further extend this concept by combining cartilage modules with different states to keep region-specific functions after assembly, which is especially relevant for interface tissues.[110]

However, despite their high biological fidelity, self-assembled organoids face intrinsic limitations that hinder their standalone clinical application. A primary drawback is the lack of precise structural control. Cellular self-organization and tissue growth can often lead to variable heterogeneous geometries that may not match patient-specific anatomical defects. Additionally, these constructs are constrained by diffusion limits, leading to necrotic cores in larger assemblies due to insufficient nutrient and oxygen transport.[171] Finally, purely cellular aggregates generally lack the immediate mechanical integrity required to withstand physiological loading in load-bearing bone defects, necessitating their combination with structural reinforcement strategies.[172]

6.2. Field-assisted assembly strategies

Magnetic fields enable contactless four-dimensional biofabrication by guiding magnetized cells or microtissues into predefined architectures and promoting their fusion without changing the culture chemistry.[173,174] Importantly, time-varying fields can also provide controlled mechanical actuation—often mimicking physiological gait cycles—to steer maturation programs relevant to endochondral repair.[175,176] In callus-inspired constructs, magnetically augmented callus assembloids were assembled and mechanically programmed, and this was linked to activation of mechanosensitive Hippo and Wnt pathways and faster ECO *in vivo*.[176] Remote magnetic stimulation of engineered tissues has likewise been shown to enhance bone regeneration by triggering focal adhesion kinase and mitogen-activated protein kinase signaling, supporting magnetic actuation as a practical lever for both construct formation and post-assembly maturation.[177] A key limitation is the need to standardize magnetic loading and field delivery across scales to avoid heterogeneity and off-target mechanical dosing. Acoustic field-based methodologies have demonstrated promise as non-contact tools for spheroid formation and 3D sample manipulation.[178,179] Nonetheless, their specific application in callus organoid engineering remains unreported.

6.3. Biofabrication strategies

6.3.1. Extrusion-based bioprinting

Extrusion-based bioprinting remains the most widely used biofabrication approach due to its ability to process high-viscosity bioinks and high cell densities, which are essential for creating scalable tissue constructs.[160,180,181] In organoid-driven bone repair, extrusion is primarily used for spatial patterning to position and space microtissue modules with defined local density and geometry while preserving void space for diffusion and later integration. The same workflow also imposes microtissue-specific constraints: printing commonly relies on support-bath rheology and rapid stabilization, whereas tissue performance depends on maintaining low shear and preserving aggregate cohesion.[182]

A particularly useful extension of organoid printing is embedded (support-bath) printing, in which soft inks or spheroids are deposited into a yield-stress bath that holds them in place until stabilization. Freeform Reversible Embedding of Suspended Hydrogels is a widely cited example of a reversible support bath that enables the printing of very soft hydrogels that would otherwise collapse.[183] More directly relevant to modular tissue construction, Daly *et al.*[159] demonstrated high-resolution transfer and patterning of spheroids within self-healing support hydrogels, followed by controlled spheroid fusion into organized, high cell-density microtissues. Overall, for callus organoid concepts, extrusion is most valuable for standardizing spatial organization and fusion geometry, while the developmental maturation of the module remains the main driver of tissue progression.

6.3.2. Light- and laser-assisted biofabrication

Light-based techniques, such as digital light processing, stereolithography, and volumetric bioprinting, are typically selected when shape fidelity and fine architectural features are priorities, for example, to match complex geometries or introduce defined pores/channels that can support transport and invasion. The main constraint is photochemistry: polymer composition, photoinitiator choice, and exposure dose must be tuned to achieve curing while maintaining cell function in cell-laden formulations.[184]

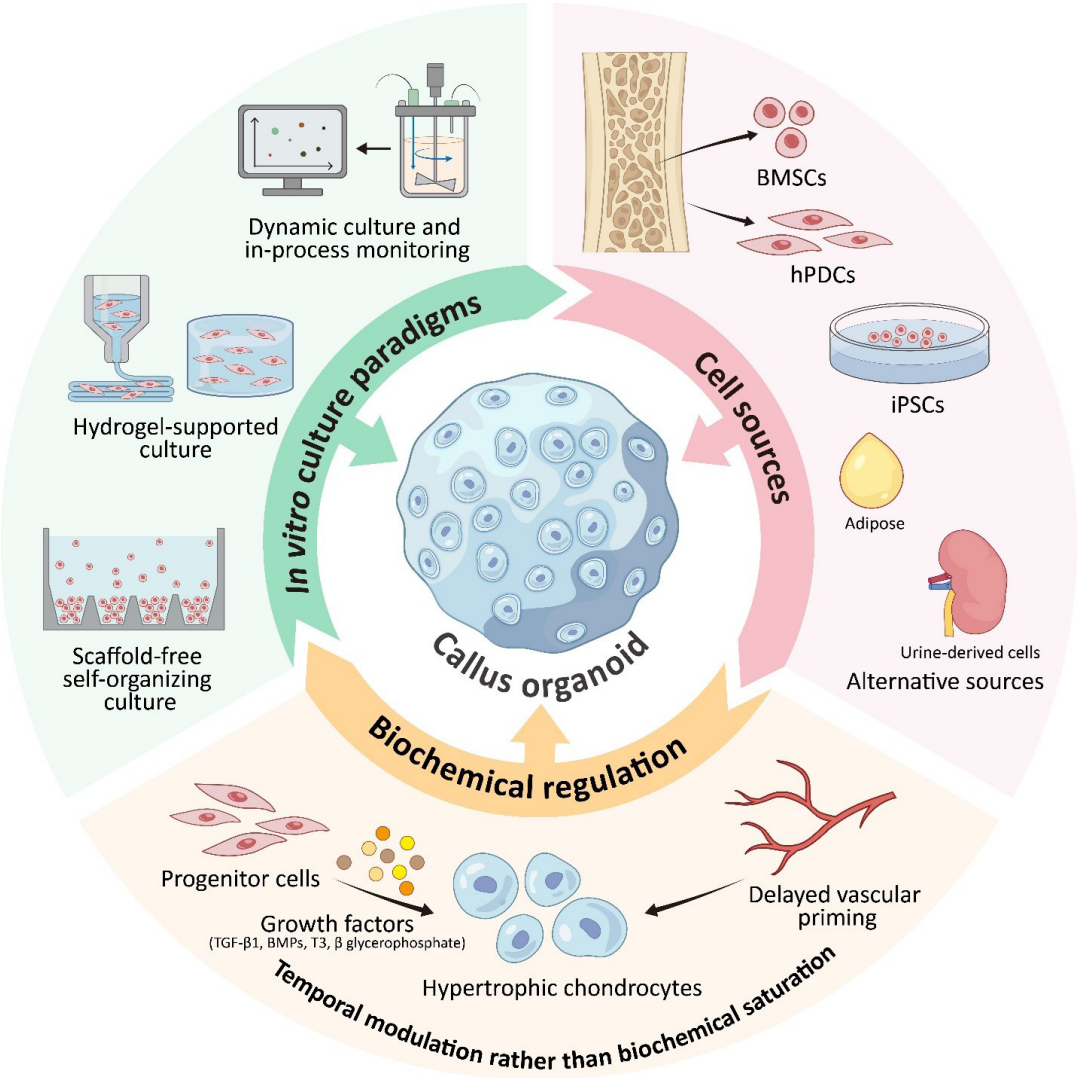

Figure 4. Overview of major design dimensions for callus organoid construction.
Abbreviations: BMPs: Bone morphogenetic proteins; BMSCs: Bone marrow mesenchymal stromal/stem cells; hPDCs: Human periosteum-derived progenitor cells; iPSCs: Induced pluripotent stem cells; T3: Triiodothyronine; TGF: Transforming growth factor. Image created by the authors using Adobe Illustrator, 2026 version (Adobe Inc., USA).

Recent studies have used projection stereolithography to build perfusable channel networks in hydrogels, showing how transport-relevant architecture can be designed into thick constructs.[185] Tomographic volumetric bioprinting offers another route, producing 3D constructs rapidly and shifting the main limitation from printing time to bioresin design and optical control.[186] In addition, laser-assisted bioprinting is mainly used for the precise patterning of cells and biomaterials when nozzle-based printing is limited.[187,188] In organoid-based skeletal repair, these approaches are most often used to build carriers or architectures that control shape and transport, while module maturation and remodeling remain biology-driven.

6.3.3. Fiber-based scaffolding: Electrospinning and melt electrowriting

Fiber-based scaffolds are frequently used to provide early stability and geometric confinement around microtissues while remaining permissive to invasion and remodeling. Electrospinning is a classic method for producing ECM-like fibrous materials and is widely recognized as a versatile approach for generating micro/nanofiber networks.[189] A well-recognized limitation is that electrospun fibrous meshes are typically composed of closely packed nanofibers, which can yield only superficial porosity, thereby hindering cell infiltration and tissue ingrowth unless pore architecture is deliberately engineered.[190] Consistent with the broader design logic of barrier membranes in guided

bone regeneration, such electrospun layers are therefore most commonly deployed as thin wraps, membranes, or interface components that stabilize the defect space and exclude fast-migrating soft tissues, rather than serving as the sole 3D structural framework.[191]

Melt electrowriting (MEW) fundamentally differs from conventional electrospinning in the scale of fibers it produces, typically generating stable micrometer-scale fibers rather than nano or submicron fibrous meshes.[192] The larger and more uniform fiber diameter, combined with the precision of the writing process, facilitates more predictable fiber placement and the creation of well-defined pore architectures.[193] A widely cited review by Dalton[194] describes MEW as an additive-manufacturing-aligned writing technology where controlled fiber placement is an important engineering advantage. Reviews also emphasize that MEW fiber architecture strongly influences macroscopic mechanical behavior and can be tuned through design choices, while polymer selection and processing constraints define the feasible design space.[133] From the perspective of modular skeletal repair, MEW is therefore often used as a cage/mesh to hold microtissues in place, standardize packing, and preserve void space for later invasion. For instance, a recent study designed MEW-assembloids by combining the biological potency of cartilaginous microtissues with the structural stability of tubular MEW meshes, thereby preventing soft-tissue collapse in critical-sized defects while supporting ECO.[66] Overall, electrospinning mainly provides fibrous interface support, whereas MEW provides a defined micro-architecture for early stability; both act as reinforcement that supports organoid-driven remodeling.

6.4. Injectable and minimally invasive delivery strategies

Injectable delivery is attractive for bone defects when surgical access is limited or the defect area is irregular. In most studies, injectable hydrogels are used as local carriers as they can fill the defect space, stabilize *in situ* via physical or chemical crosslinking, and retain therapeutic cargo (proteins, small molecules, extracellular vesicles, or cells) at the injury site. A core design requirement is to balance injectability, including protection of encapsulated cells or microtissues during delivery, with post-injection stability to reduce washout and enable early host invasion.[195]

For developmental engineering and callus organoid strategies, the carrier is most useful when it supports the delivery of living modules without locking them into a non-remodeling state. Hydrogels built on dynamic/reversible crosslinks provide one practical route: they can flow under shear during injection and then recover their network afterwards, improving retention while remaining cell-infiltratable. A representative example is the "dynamic and cell-infiltratable" injectable hydrogel platform reported for challenging bone defects, which emphasizes injectability and permissiveness for cell entry and remodeling rather than permanent encapsulation.[196]

Recent work also illustrates how injectable systems can be engineered to address compromised healing environments. In diabetic bone regeneration, a multistimuli-responsive double-network hydrogel was designed to release interleukin-10 and BMP-2 in a cue-linked manner and was reported to improve repair through immunomodulatory effects.[197] In infected bone defects, a biomimetic methacrylated collagen hydrogel combined with composite native bone inorganic salts and antibacterial microspheres (loaded with BMSCs) was reported to support rapid vascularized and innervated bone regeneration in a rabbit infected bone defect model, illustrating how injectable formulations can couple antimicrobial function with an osteoinductive microenvironment.[198] Overall, injectable strategies are best viewed as a way to place and retain bioactive cargo or living modules in complex defects, while tuning gelation, transport, and remodeling permissiveness so that vascular-coupled bone formation can proceed *in vivo*.

6.5. Extracellular matrix-based strategies

To mitigate the practical bottlenecks of living callus organoid implants, including limited shelf life, manufacturing-to-delivery logistics, and the added immunological burden of allogeneic cell products, recent studies have increasingly reframed endochondral repair as an off-the-shelf, ECM-instructive strategy, leveraging devitalized/decellularized cartilage templates that preserve soft-callus-like cues while removing viable cells.[199,200] This direction aligns with broader ECM-biomaterials principles in regenerative medicine, where decellularization is used to retain tissue-specific instructive signals while reducing cell-associated risks, improving standardization and deployability.[201,202] Cunniffe *et al.*[203] engineered a porous scaffold from decellularized hypertrophic cartilage, hypothesizing that the preserved matrix could recruit host cells and support endochondral regeneration in large defects. Pigeot *et al.*[204] used apoptosis-induced devitalization of human MSC-derived hypertrophic cartilage and showed, in a rabbit calvaria model, that the acellular matrix outperformed a clinical bone substitute in bone volume formation. Their subsequent study introduced BMP-2-programmed hypertrophic cartilage constructs that demonstrated "unprecedented osteoinductivity," exceeding both BMP-2 protein delivery and living-cell templates.[199] Longoni *et al.*[205] developed allogeneic devitalized soft-callus spheroids and observed full bridging of rat femoral defects within four weeks. De Silva *et al.*[206] extended this strategy to a large-animal model, showing that modular devitalized

callus-mimetic implants restored critical-sized defects in goats with outcomes comparable to autografts. These findings suggest that devitalized hypertrophic cartilage ECM provides potent angiogenic and osteoinductive signals even in the absence of viable cells. Bourgine *et al.*[112] demonstrated that apoptosis-based devitalization preserves GAGs and matrix-bound factors essential for vascular invasion and remodeling, which are often lost in harsh decellularization methods. Notably, decellularized cartilage matrices can modulate host immunity: Garcia-Garcia *et al.*[200] showed that cell-free hypertrophic cartilage grafts promote anti-inflammatory macrophage and dendritic cell phenotypes and suppress T-cell activation, enabling healing even in immunocompetent settings. Taken together, these studies raise a critical question: do callus organoids truly require living cells to achieve bone regeneration, or is their ECM-derived microenvironment alone sufficient to orchestrate full repair?

Overall, these different engineering strategies, including self-assembled living modules, field-assisted assembly, biofabrication, injectable delivery, and ECM-based approaches, are summarized schematically in Figure 5.

7. Bottlenecks and challenges

7.1. Donor variability and biological heterogeneity

A major practical challenge, not only in callus organoid engineering but across tissue engineering and the broader organoid field, is the inherent variability of primary cell sources, which directly undermines the reproducibility of callus organoids.[207] Because these constructs are designed under developmental engineering principles—where early fate decisions propagate through staged, non-linear biological cascades—donor-specific differences in progenitor composition and differentiation dynamics can translate into distinct maturation trajectories and variable potency.[18,208] Recent evidence indicates that even among bone-forming callus organoids, donors may preferentially follow divergent ossification routes, including a canonical (pre)hypertrophic cartilage program or a fibrocartilaginous intermediate.[18] This divergence appears biased by biological sex in hPDCs, with male donors more frequently generating hypertrophic, matrix-rich organoids, whereas female donors are more often associated with a fibrocartilaginous phenotype characterized by limited matrix maturation and lower tissue yield.[18] Donor age further modulates progenitor competence; for example, an age-related decline in TGF-β-driven chondrogenesis has been reported in male BMSCs.[209] Beyond intrinsic traits, donors can also differ markedly in their responsiveness to molecular modulation (e.g., miRNA inhibition), resulting in "responder" versus "non-responder" phenotypes that reflect dependence on baseline state.[210] Importantly, conventional two-dimensional assays and pellet readouts do not reliably predict differentiation performance in 3D biomaterial contexts, motivating the development of standardized, non-destructive potency assays and release-relevant critical quality attributes.[18,207] Ensuring functional consistency across donors and manufacturing batches, therefore, remains a key bottleneck for regulatory translation and clinical implementation of organoid-based living implants.[207]

7.2. Mass transport limitations

In 3D bone and cartilage tissue engineering, diffusion-limited transport of oxygen and nutrients becomes a dominant design constraint as constructs approach clinically relevant dimensions. Quantitative diffusion-reaction models set practical limits on oxygen penetration and construct size, and show how architecture shapes viability and patterned phenotypes.[9,211] Similar metabolic limits are documented in bone and cartilage organoids: under static conditions, passive diffusion typically sustains supply only over 100–200 μm from a well-perfused interface, and hypoxic or necrotic cores emerge as diameter increases.[172]

Early work by Malda *et al.*[212] demonstrated intra-construct oxygen gradients in polyethylene glycol terephthalate/polybutylene terephthalate cartilaginous scaffolds and fitted diffusion-reaction models to estimate effective oxygen diffusivity and cellular consumption. They reported an oxygen diffusion coefficient in native cartilage of ~2.2×10^{-9} $m^2 s^{-1}$ (≈71% of water), while the polymer was ~25-fold lower, underscoring scaffold microstructure and interconnected porosity as major transport bottlenecks.[212] Similarly, Figueiredo *et al.*[213] showed that increasing polymer content in silated hydroxypropyl methylcellulose hydrogels reduced effective oxygen diffusivity and produced core depletion during culture.

Scaffold-free aggregates help establish the canonical "viable shell/hypoxic core" paradigm. In chondrocyte pellets, Li *et al.*[214] combined oxygen uptake measurements with diffusion-reaction modeling and identified a threshold of oxygen partial pressure around ~8% of atmospheric pressure: below this level, proteoglycan accumulation increased, whereas more oxygenated regions favored collagen synthesis. Likewise, Lewis *et al.*[215] attributed heterogeneous proliferation to spatial oxygen gradients, with a proliferative outer shell surrounding a hypoxic, poorly proliferative core in larger constructs. Together, these studies established the shell–core paradigm (pellets/aggregates up to ~2 mm) and linked oxygen ranges to chondrogenic outcomes, and similar architectures are frequently observed in avascular bone and cartilage organoids when diameters exceed 0.5–1.0 mm under static culture.[215,216]

Encapsulated MSC systems further demonstrate that diffusion limitation is intrinsically time-dependent, as oxygen demand varies with cell state. Carroll *et al.*[217] measured central oxygen levels in agarose hydrogels seeded with BMSCs, infrapatellar fat pad MSCs, or chondrocytes and used numerical modeling to infer spatial gradients and construct-level consumption. Undifferentiated MSCs displayed higher initial oxygen consumption that decreased with chondrogenic differentiation toward a more chondrocyte-like phenotype, implying that early near-anoxic regions can partially relax over time even without changes in geometry or boundary oxygen.[217] More generally, these data underscore the need to parameterize diffusion-reaction models with cell-source- and stage-specific oxygen consumption, rather than constant monolayer-derived values. For bone and osteochondral organoids, time-resolved oxygen consumption is increasingly incorporated into organoid-scale mass-transport models.[218,219]

Construct size and geometry are likewise critical. Buckley *et al.*[220] systematically varied the dimensions of MSC-laden agarose cylinders from Ø4 × 1.5 mm to Ø8 × 4.5 mm and found that total sulfated GAG and collagen contents, as well as dynamic modulus, increased with construct scale over 42 days. Although intra-construct oxygen was not directly measured, the improvement in bulk mechanical function with increasing size is consistent with moderately hypoxic interior regions that remain viable and anabolic for cartilage-like matrix deposition.[214,220] Daly *et al.*[221] further demonstrated that both construct size and external oxygen tension modulate the balance between diffusion limitation and chondrogenic potential in MSC alginate hydrogels: under static culture at 20% oxygen, large constructs developed central regions devoid of matrix, whereas dynamic bioreactor culture at 3% oxygen supported the formation of large, spatially more homogeneous cartilaginous tissues. These findings reinforce that oxygen gradients are not uniformly detrimental; their magnitude and spatial distribution can be tuned via geometry and boundary conditions to favor desired matrix patterns. In organoid systems, this has motivated the use of gradient microenvironments, for example, exploiting the natural transition from avascular hypoxic cartilage to vascularized subchondral bone to guide single BMSC-derived cartilage organoids toward heterogeneous osteochondral structures *in vivo*.[222]

Perfusion through macroporous scaffolds can reduce external mass-transfer resistance and maintain near-normoxic conditions across millimeter-thick constructs compared to static culture, which often yields peripheral shells.[223–226] Experimental analyses, however, show that simply increasing flow is insufficient: higher perfusion improves core oxygenation but simultaneously elevates shear stresses at scaffold surfaces, which can impair viability.[223,227] More advanced designs introduce perfusable channels within hydrogels or porous scaffolds to reduce the effective diffusion path length. In such systems, oxygen diffusion from channels into surrounding tissue has been modeled to determine channel spacing that maintains adequate oxygenation in scaled-up constructs, calibrated by measured diffusion coefficients and spatial mineralization patterns.[227,228] These data are broadly consistent with characteristic oxygen penetration distances of 100–250 μm from a well-supplied interface in dense tissue, implying that channel half spacing and construct radii must be selected with this length scale in mind.

Collectively, these studies offer key parameters for realistic diffusion-reaction modeling in chondrogenic and osteogenic constructs, emphasizing that transport properties evolve over time due to ongoing matrix deposition, crosslinking, and mineralization, thereby altering effective diffusivity during long-term culture.

7.3. Incomplete tissue complexity

Fracture healing is not a simple "bone-forming" process but rather a highly complex series of events orchestrated by the coordinated action of blood vessels, nerves, muscle, lymphatics, and immune factors. Vascularization is central to this process: beyond delivering oxygen and nutrients, blood vessels also act as active signaling hubs that secrete angiogenic factors, thereby shaping the cellular programs of repair.[229,230] Specialized H-type vessels provide key paracrine cues that guide osteoprogenitor cell proliferation, migration, and lineage commitment, coordinating angiogenesis with bone formation.[229,230] At the same time, peripheral sensory nerves, especially *TrkA*$^+$ fibers, respond rapidly to injury, and neurotrophic signals such as fibroblast growth factor-9 can directly promote bone regeneration; conversely, loss of neural input is associated with markedly reduced callus formation and compromised healing.[231,232] In addition, skeletal muscle adjacent to the fracture harbors *Prx1*$^+$ mesenchymal progenitors that contribute to early repair by driving an initial fibrotic response and by directly supplying cells to cartilage and new bone formation.[133] Recent work has further highlighted the multifaceted roles of the lymphatic system in bone repair: lymphatic drainage can dynamically tune the inflammatory niche by clearing damage-associated molecular patterns, while lymphatic-derived factors, such as C-X-C motif chemokine ligand 12, may directly support regeneration of skeletal and hematopoietic compartments.[233,234] Within the immune cascade, macrophages function not simply as early inflammatory responders but as temporally specialized regulators of repair progression. Recent single-cell and functional studies show that fracture healing involves

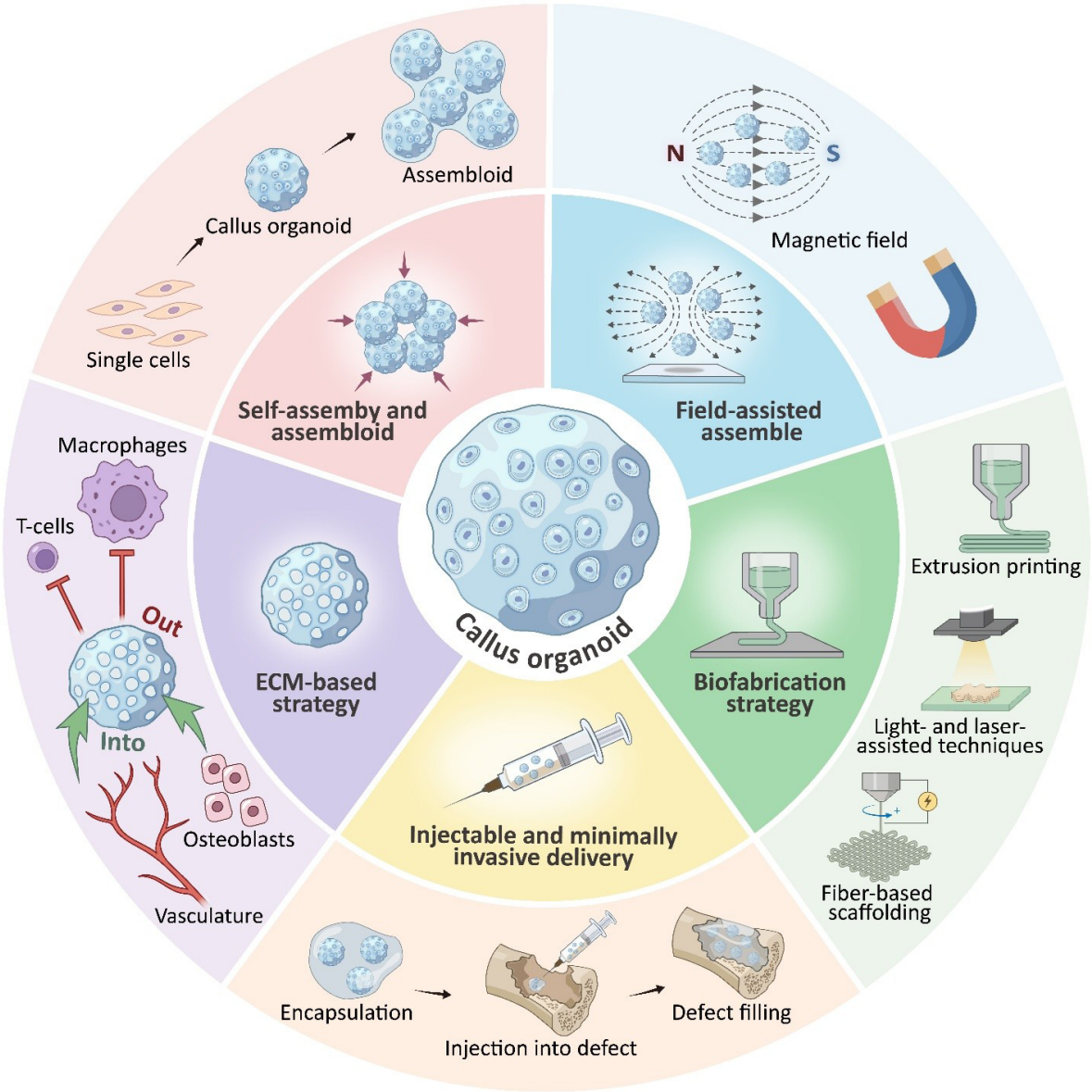

Figure 5. Schematic overview of engineering strategies for callus organoid–based tissue-engineered implants for bone defect repair. Abbreviation: ECM: Extracellular matrix. Image created by the authors using Adobe Illustrator, 2026 version (Adobe Inc., USA).

successive macrophage subsets with distinct signaling roles, coordinated in parallel with skeletal stem and progenitor cell (SSPC) state transitions; when this coordination is disrupted, prolonged pro-inflammatory dynamics can bias healing toward fibrosis and non-union.[43,45,48]

However, current callus organoids are typically generated via *in vitro* differentiation of osteoprogenitors and therefore lack the layered and coordinated contributions of vascular, neural, muscular, lymphatic, and immune components. This missing physiological complexity not only limits their fidelity as disease models but may also undermine long-term remodeling and functional stability after *in vivo* implantation.

8. Future perspectives

8.1. Vascularization and perfusion engineering

Future progress in callus organoids for skeletal defect repair will require integrating developmental engineering principles with vascularization and perfusion strategies to overcome diffusion limits and support endochondral remodeling at clinically relevant scales. As construct dimensions increase, diffusion-limited oxygen and nutrient transport become a primary constraint, necessitating engineered vascular architectures capable of sustaining coupled chondrogenic, angiogenic, and osteogenic processes.[9]

A key next frontier is the incorporation of hierarchical, perfusable vascular networks that support ECO over centimeter-scale volumes. Studies employing perfusable, endothelialized channels embedded within dense hydrogels or 3D-printed scaffolds have demonstrated significantly enhanced vascular invasion, matrix maturation, and mineralization compared to non-perfused constructs.[228,235] Moreover, 3D-printed prevascularized bone organoids, created by combining MSCs, endothelial cells, and osteogenic microparticles, can enhance *in situ* vascularized bone regeneration in critical defects, confirming the therapeutic potential of engineered microvascular networks in large bone repair.[236] Translating these approaches to callus organoids suggests hypertrophic cartilage-like microtissues pre-patterned with flow-accessible microvasculature, enabling controlled delivery of oxygen and morphogens such as VEGF, TGF-β, and BMPs that recapitulate the spatiotemporal sequence of fracture healing.[237]

In parallel, modular "bottom-up" biofabrication methods are expected to allow scalable creation of vascularized callus constructs. Prevascularized osteogenic spheroids and microtissues have demonstrated improved *in vivo* inosculation, mineralization, and functional integration compared to non-vascularized controls.[238,239] Building on this concept, callus organoids could be created from chondrogenic and osteogenic units at specific maturation stages, arranged to mimic soft callus, hard callus, and remodeling zones, with channels for adjustable flow and biochemical signals.[240,241]

Finally, precise control of vascularization will increasingly rely on integration of biofabrication with computational modeling and real-time sensing. Predictive transport and tissue-growth models have already been used to guide the design of perfusable bone scaffolds and optimize oxygen distribution.[242,243] Coupling such models with perfusion bioreactors incorporating oxygen and metabolite sensors would enable iterative refinement of vascular architectures *in vitro*. In this framework, vascularization becomes a programmable design parameter, enabling callus organoids to be tailored to defect size, anatomical location, and patient-specific conditions, thus enhancing the predictability of skeletal repair.

8.2. Advanced biofabrication techniques

In recent years, advanced biofabrication technologies have expanded the range of implant designs that can incorporate organoids. For instance, volumetric printing technology enables the high-throughput embedding of organoids in 3D configurations, thereby facilitating the fabrication of large organoid implants.[186] This technology also allows for the design of internal channels within the implants, addressing challenges related to nutrient and oxygen transport.[244] Moreover, scaffold-free or cell-only bioprinting has demonstrated the feasibility of directly printing dense cellular suspensions to form scaffold structures, potentially paving the way for the direct printing of callus tissue in the future.[245,246] It is conceivable that, in the future, callus organoids could be printed as granular bioinks for minimally invasive or *in situ* defect filling.[247] Alternatively, aspiration-assisted bioprinting may enable the precise pick-up, positioning, and assembly of spheroids or organoid modules into defined callus-like architectures.[248,249] As biofabrication technologies advance, it is anticipated that these sophisticated methods will be increasingly utilized to produce personalized bone grafts tailored to individual patients' needs.

8.3. From organoids to fracture callus systems

Current callus organoids predominantly emphasize the chondro-osseous lineage and therefore struggle to capture the systemic, stage-resolved complexity of fracture healing. A tractable path forward is to develop a modular "fracture callus system" (or fracture-on-a-chip) in which niche components mature under optimized cues and then interact through compartmentalized, programmable communication rather than being mixed from day 0.[250]

In developmental engineering, the most transferable strategy is "two-media, one-interface" control, enabling tissue-to-tissue crosstalk without compromising lineage stability. A biphasic perfusion osteochondral platform perfused chondrogenic and osteogenic media through one construct without mixing, while restricting inflammatory inputs to the cartilage compartment (either cytokine cocktails or macrophage-conditioned media) and quantifying downstream effects in the vascularized bone region.[251] This architecture directly motivates callus-system designs in which cartilage maturation, hypertrophy, and subsequent vascular invasion/ossification are driven by spatially and temporally localized inputs, rather than global medium switches.

A second enabling principle is delayed coupling: modules are matured independently, then communication is switched on to emulate stage handovers. A valve-enabled joint-on-chip demonstrated fluidic isolation during maturation followed by valve-controlled exchange, while integrating timed inflammatory priming and controlled mechanical compression.[252]

Complexity should be added pragmatically, starting with a controllable staged immune environment rather than a static inflammatory co-culture.[45,48] A useful paradigm comes from recent intestinal organoid work, where human macrophages were integrated into primary intestinal organoids to generate macrophage-augmented organoids capable of modeling not only epithelial infection

but also the resulting inflammatory response.[253] This illustrates a broader design principle relevant to callus systems: immune cells should be incorporated not merely as background components, but as functional regulators of stage progression. For fracture callus engineering, this could mean introducing macrophage inputs in a time-specific manner to recapitulate the early pro-inflammatory phase that supports SSPC activation and the later pro-repair, inflammation-resolving phase that permits cartilage-to-bone transition, rather than maintaining a fixed macrophage state throughout culture.[43,45,48] Microfluidic co-culture demonstrates that osteoblasts, chondrocytes, fibroblasts, and macrophages can be maintained in a shared microenvironment under controlled flow to reproduce paracrine dynamics.[254] Meanwhile, high-throughput microfluidic formats can generate uniform endothelial-containing bone organoids, supporting scalable assembly and batch consistency.[255] Importantly, joint-on-chip practice cautions that added compartments can erode throughput and reproducibility, demanding a measured balance between physiological breadth and tractability.[256] Controlled perfusion/loading regimes that enhance osteogenesis further strengthen mechanobiological relevance.[257] In short, stage-aware modularity, rather than maximal complexity, will be the key to building tractable, translational fracture callus systems.

9. Conclusion

Callus organoids reframe bone defect regeneration as the development of tissue implants that can undergo programmed transitions linked to bone regeneration rather than a mimicry of a fully mature bone substitute. Rooted in developmental engineering, they aim to recapitulate the fracture callus as a transient, instructive tissue that can withstand early avascular conditions, promote vascular entry, and then remodel into bone *in vivo*. This perspective also sharpens what should be engineered and compared across studies: trajectory position at implantation and handoff capacity to the next repair phase.

Strategies for callus organoid engineering converge on one goal: engineering a controllable endochondral starting state through coordinated biochemical and biophysical programming, rather than relying on a single "best" cell type or recipe. Across tissue-aligned progenitors (e.g., periosteum- and marrow-derived cells) and pluripotent sources, modular microtissues are guided with time-ordered cues for chondrogenesis, hypertrophy, and pro-vascular/osteogenic handoff, together with 3D context and assembly architecture. The take-home message is that robustness is achieved by managing the "callus" intermediate state, which includes the maturation stage, cell phenotype, and matrix context. Factors such as cell source, priming history, cue timing and dose, and microtissue organization help standardize constructs for comparable outcomes. Key bottlenecks remain, including donor variability, mass transport limitations, and incomplete tissue complexity, and these point to three priorities. Vascularization/perfusion can relieve diffusion constraints and improve host integration; advanced biofabrication can scale up and standardize constructs by controlling final implant size and architecture. Moving from organoids to fracture callus systems will prioritize stage-coupled platforms that integrate vascular/immune components and assess biological phase transitions rather than static endpoints. In parallel, ECM-based strategies (devitalized or decellularized callus-like templates) may preserve instructive cues in an off-the-shelf format.

Overall, the goal in the coming years is not a perfect "bone organ *in vitro*," but a standardized callus-like transient tissue that reliably executes endochondral repair after bone defect implantation, thereby illustrating a broader paradigm in which organoids are engineered to recapitulate physiological or pathological processes for tissue engineering and regenerative medicine.

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Conflict of interest

The authors declare they have no competing interests.

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Further disclosure

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