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Turning Nature's own processes into design strategies for living bone implant biomanufacturing: a decade of Developmental Engineering

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ABSTRACT

A decade after the term developmental engineering (DE) was coined to indicate the use of developmental processes as blueprints for the design and development of engineered living implants, a myriad of proof-of-concept studies demonstrate the potential of this approach in small animal models. This review provides an overview of DE work, focusing on applications in bone regeneration. Enabling technologies allow to quantify the distance between *in vitro* processes and their developmental counterpart, as well as to design strategies to reduce that distance. By embedding Nature's robust mechanisms of action in engineered constructs, predictive large animal data and subsequent positive clinical outcomes can be gradually achieved. To this end, the development of next generation biofabrication technologies should provide the necessary scale and precision for robust living bone implant biomanufacturing.

Contents

Abbreviations: ATMP, Advanced Therapeutic Medicinal Product; DE, developmental engineering; TE, tissue engineering; EO, endochondral ossification; ECM, extracellular matrix; MSC, mesenchymal stromal cell; BM-MSC, bone marrow derived MSC; PDC (hPDC, mPDC), periosteum derived cell (h: human (m: mouse)); ADSC, adipose derived stem cells; scRNA-seq, single cell RNA sequencing.; ABG, autologous bone graft; hUCB-BFs, human umbilical cord blood-borne fibroblasts; mESC, mouse embryonic stem cells; AC, articular chondrocytes; TCP, tricalcium phosphate; CDM, cartilage-derived matrix; PEG, poly(ethylene glycol); PLGA, Poly Lactic-co-Glycolic Acid; PCL, polycaprolactone.; CHyA, collagen hyaluronic acid; CQA, critical quality attribute; GMP, good manufacturing processes; CoG, cost of goods; QbD, quality by Design.

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1. The concept

The classical tissue engineering (TE) paradigm seeks to develop living implants for repairing, maintaining or replacing damaged or missing tissue and organs. These living implants are formed by combining cells, scaffolds and/or chemical stimuli. After almost four decades, many clinical trials are ongoing involving tissue engineered products, however tangible results in terms of commercially available products are still rather limited [1]. After the initial TE hype died down at the end of the 20th century, the field matured into a proper scientific discipline. In order for the scientific advances to be translated into robust clinical therapies, the TE field is currently undergoing yet another transformation. Aspects such as manufacturing, regulatory approval and industrial uptake are considered at increasingly early stages in the R&D process.

A little over a decade ago, a paradigm shift was proposed from classical TE towards developmental engineering (DE), taking developmental processes as blueprints for regeneration of tissue and organs [2–6]. A schematic representation of both classical and DE strategies in the bone TE field is given in Fig. 1 and explained in the following sections. Developmental cascades are tightly regulated, semi-autonomous and robust. Following a biomimetic strategy holds the promise of recapitulating not only the successful formation of tissues and organs of interest, but doing it by recapitulating the desirable process-related traits including regulation, autonomy and robustness. Since the term 'developmental engineering' was coined a little over a decade ago, research on this strategy has boomed in all organ systems.

This review addresses the basic concepts of DE and shows how adhering to DE principles leads to powerful biological results. An overview is provided of multi-omics technologies able to provide detailed information on both the biological blueprint and the regenerated tissue. In addition, systems biology approaches are discussed that allow transforming this information into actionable knowledge. Subsequently it discusses how enabling technologies such as biomanufacturing and automation can be tailored for DE applications and how the previously presented characterization technologies can be transformed into quality controls during production. The review ends by arguing how following a DE strategy might facilitate robust translation to clinical applications. Bone tissue engineering and regeneration of large skeletal defects are used throughout the text as a case study.

2. To the bone

Bones in the body develop through two distinct processes. The flat bones, *e.g.* of the skull, develop via intramembranous ossification where mesenchymal condensation is followed by differentiation into osteoblasts, which form bone. However, the majority of bones, *i.e.* long bones, develop through endochondral ossification (EO) where mesenchymal cells condense and differentiate into chondrocytes to create a cartilage template. The chondrocytes mature into hypertrophic chondrocytes and secrete metalloproteinases, which degrade the collagenous extracellular matrix (ECM), and growth factors (*e.g.* VEGF) which attract blood vessels. The mature cartilage template is subsequently remodeled into bone while growth plates form at the bone ends (Fig. 1, central right). The growth plates consist of a gradient of chondrocytes, with specific gene signatures, from proliferative (*Sox9*, *Col2a1*), prehypertrophic (*Sox9*, *Foxa2/3*, *Mef2c*, *Ihh*) to hypertrophic

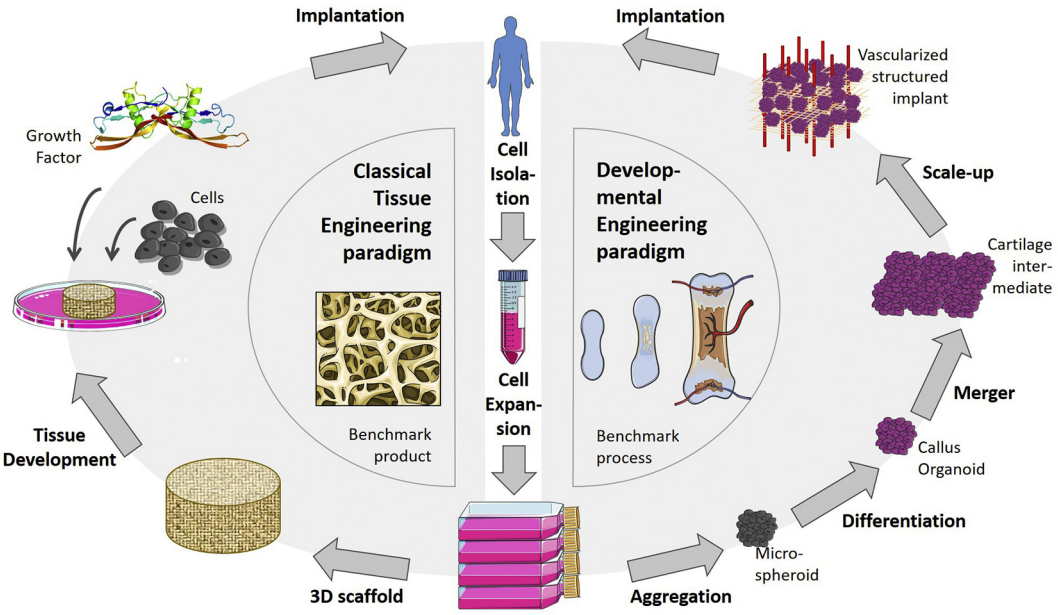

Fig. 1. Schematic overview of the classical Tissue Engineering paradigm versus the Developmental Engineering paradigm, along with their biological blueprint, using bone regeneration as an example.

(*Runx2/3*, *Sp7*, *Mmp13*, *Spp1*) chondrocytes which drive the longitudinal bone growth [7]. Interestingly, recapitulation of the EO process also occurs during fracture healing with the formation of a cartilaginous callus [8]. Recapitulation of EO has therefore been suggested as a promising DE strategy for treating long bone defects through the creation of cell-based cartilage intermediate constructs [6,9–11].

The last decade, we saw a drastic increase in the amount of studies where *in vitro* engineered DE constructs were able to form bone structures as well as bone organs upon *in vivo* ectopic implantation in the absence of additional growth factors such as BMPs and TGFs (Fig. 2A and Table 1). Recently, this has also been shown for challenging long-bone defects (Table 2) [12–14] indicating that the DE strategy paves the way for establishing an unprecedented link between engineered implant quality attributes *(in vitro)* and final outcome upon implantation. This link is of paramount importance since it may allow the development of predictive potency assays and hence enable the implementation of Quality by Design for DE implant manufacturing.

The advantage of the DE-EO approach as compared to the intramembranous ossification (direct bone formation) strategy has been demonstrated by chondrogenic differentiation of bone marrow-derived mesenchymal stromal cells (BM-MSCs) seeded on calcium and/or phosphate-based scaffolds [15–17] (Fig. 2B). However, a common approach to recapitulate the cellular condensation process during endochondral skeletal development is to seed hundreds of thousands of cells that aggregate and form millimeter sized scaffold-free pellets or micromasses [18–25]. This strategy has further been developed by differentiation of micro-sized spheroids (−100 μm in diameter) to tackle difficulties of diffusion limitations and modularity [26]. Spheroids differentiate into bone forming units ("callus organoids" or "microtissues") which, upon assembly, form bone organs without fibrotic tissue both ectopically and in long bone defects [12] (Fig. 2C). Besides spheroids [12], the DE building blocks can be produced in the form of cell sheets [13,14], tissue strands [148] or pellets [24,26,149].

Experiments in long bone defects have also demonstrated the importance of (additional) mechanical stimulation to improve the outcome of endochondral ossification (Fig. 2D) [13,14]. The combination of the above described advancements with emerging biofabrication techniques is expected to move the DE-EO approach towards robust manufacturing processes with scale-up possibilities (Fig. 2E) [27,28]. The DE-EO approach for bone TE has been reviewed elsewhere in more detail, focusing on different aspects such as endochondral priming [6], native ECM biomaterials [29], cell spheroids [30], craniomaxillofacial bone regeneration [31] and biomaterials [32–34].

Despite successful bone formation through EO, variable results have been seen in orthotopic experiments and additional advancements are required to bring endochondral bone tissue engineering towards preclinical studies with large animals and a robust advanced therapy medicinal product (ATMP) (discussed in section 4). Furthermore, current *in vitro* quality controls linked with *in vivo* bone forming potential have mainly included bulk gene expression (qPCR, bulk RNA sequencing) which only generates an average gene expression of all cells within the constructs. In addition, these methods are destructive and are therefore not optimal as potency indicators for a manufacturing process. A combination of omics-approaches, *i.e.* transcriptomics (discussed in 3.1) and metabolomics (discussed in 3.3), might be important in defining differentiation maturity *in vitro*, in parallel with increased

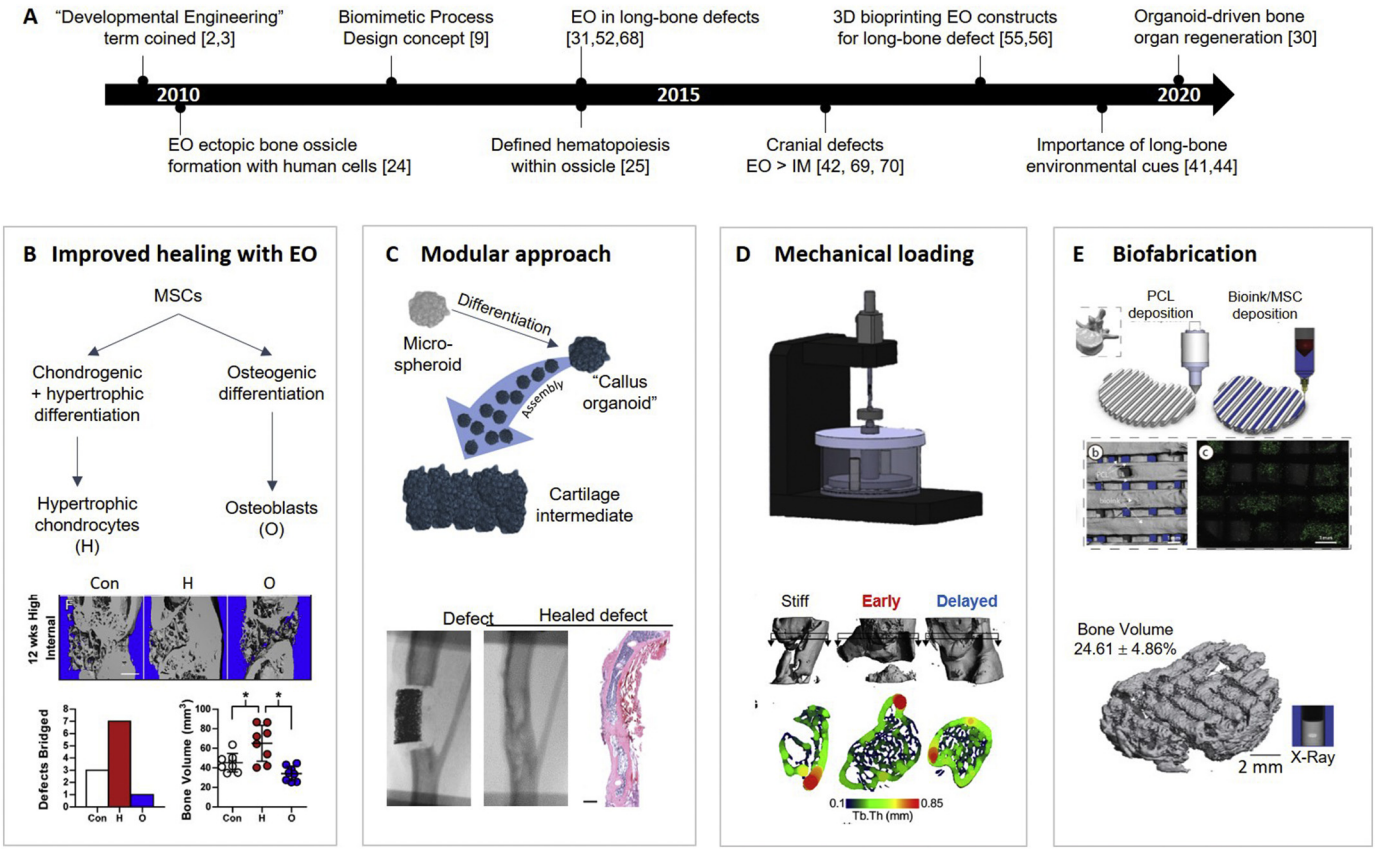

Fig. 2. A decade of Developmental Engineering in Bone Tissue Engineering. (A) Timeline of important perspective and proof-of-concept papers. (B) Improved bone formation through endochondral ossification (EO) as compared to intramembranous ossification (IM) in a critical-size long bone defect in rat. Con: acellular scaffold, H: hypertrophic chondrocytes, O: osteoblasts [17]. (C) Modular approach with "callus organoids" heals murine critical-size long bone defects [12]. (D) Mechanical stimulation affects differentiation *in vitro* and *in vivo*. Local trabecular thickness mapping illustrates that stabilizing long bone defects results in less bone formation compared to less stable defects (early/delayed loading) [14]. (E) Advancements in biofabrication tools open the possibility for formation of larger and more complex structures [27].

Table 1
Tissue Engineered constructs for endochondral bone formation upon ectopic implantation. Qualitative *in vivo* output defined at the final time point with presence or not of FT: fibrotic tissue, C: cartilage, B: bone and BM: bone marrow. BM-MSCs: human bone marrow-derived mesenchymal stromal cells, hUCB-BFs: human umbilical cord blood-borne fibroblasts, hPDCs: human periosteum-derived cells, hASCs: human adipose-derived stem cells, mESC: mouse embryonic stem cells, AC: articular chondrocytes, TCP: tricalcium phosphate, CDM: cartilage-derived matrix, PEG: poly(ethylene glycol), PLGA: Poly Lactic-co-Glycolic Acid, PCL: polycaprolactone.

Cells	Culture set-up/ Biomaterial	Cell density	Chondrogenic media	Hypertrophic media	*In vivo*	*In vivo* output	Reference
ATDC5	Pellet	2 x 10^5 cells/pellet	3 weeks	/	4, 8 weeks	C, B	[25]
hBM-MSCs	Pellet	5 x 10^5 cells/pellet	3-7 weeks	/	4-6 weeks	C, B	[18]
hBM-MSCs	Pellet	2 x 10^5 cells/pellet	4 weeks	/	8 weeks	C, B, BM	[19]
hBM-MSCs	Pellet	3 x 10^5 cells/pellet	4 or 3 weeks	0 or 2 weeks	8 weeks	B, BM	[22]
hBM-MSCs	Pellet	2 x 10^5 cells/pellet	4 weeks	/	4 weeks	FT, B	[20]
hBM-MSCs + monocytes	Pellet	5 × 10^5 cells/pellet	3 weeks	2 weeks	3, 8 weeks	FT, C, B	[23]
hUCB-BFs	Pellet	3 x 10^5 cells/pellet	3 weeks	/	8 weeks	B, BM	[194]
hPDCs	Micromass	4 x 10^5 cells/micromass	4 weeks	/	8 weeks	FT, B, BM	[24]
hPDCs	Spheroid	250 cells/spheroid, 3000 spheroids/implant	3 weeks	/	4, 8 weeks	B, BM	[12]
hBM-MSCs	Transwell	5 x 10^5 cell/sheet	3w serum-free chondrogenic	2 weeks	4, 8 weeks	B, BM	[21]
hBM-MSCs	Pellet + alginate + PCL nanofiber mesh	2.5 x 10^5 cells/pellet	3 weeks (+ 3 weeks endothelial media)	/	4 weeks	S, C	[195]
hASCs	1.2% alginate (w/v) + agarose	Pellet: 250 000 cells; alginate beads: 8 x 10^6 cells/mL	4 weeks	/	8 weeks	FT, B	[196]
Porcine BM-MSCs	Alginate/fibrin/chitosan hydrogel	20 x 10^6 MSCs/ml	5 weeks	1 week	6 weeks	C, B, BM	[197,198]
hBM-MSCs	Bovine collagen type I + chondroitin sulphate	1 x 10^6 cells/scaffold	4 weeks	/	8 weeks	B, BM	[19]
hBM-MSCs	b-tricalcium-phosphate (β -TCP) + fibrin	5 x 10^5 cells/construct	6 weeks	/	8 weeks	C, B, BM	[16]
mESCs/hBM-MSCs	Calcium phosphate ceramic	5 x 10^5 cells/scaffold	3 weeks	/	3 weeks	FT, B	[199]
chick chondrocytes	Chitosan sponge	16 x 10^5 cells/ml	3 weeks	1 week	5 months	C, B, BM	[15]
equine BM-MSCs + chondrocytes	GelMA + equine CDM particles	20 x 10^6 cells/ml	2 weeks	/	8 weeks	C, B, BM	[200]
hBM-MSCs	Human fibrin	10 000 cells/spheroid, 60 micropellets per 100ul	1 or 4 weeks	/	10-13 weeks	B, BM	[26]
Human fractionated adipose	Human fractionated adipose, cultured 3 weeks		4 weeks	2 weeks	8 weeks	B, BM	[201]
hBM-MSCs	Matrigel-equivalent matrix	6.7 x 10^6 cells/ml	/	/	2, 4, 6, 8-12 weeks	B, BM	[202]
hBM-MSCs	Matrigel-equivalent matrix	6.7 x 10^6 cells/ml	/	/	8 weeks	B, BM	[202,203]
hASCs	None/type I collagen-based scaffold	5 x 10^5 cells/pellet; 40 x 10^6 cells/scaffold	4 weeks	w/wo 2 weeks	8 weeks	B, BM	[204]
hPDCs	NuOss™	47 x 10^6 cells/ml	/	/	4, 8 weeks	FT, B	[205]
hBM-MSCs/hNasal chondrocytes	PEG hydrogel + TGFβ	20-25 x 10^6 cells/ml	2 days?	/	2, 4, 8, 12 weeks	B, BM	[206]
hBM-MSCs	PET fibrous mesh	66 × 10^6 cells/ml	2 weeks	1 week	2, 4, 8 weeks	-	[207]
rat BM-MSCs	PLGA/PCL electrospun or HA/TCP	1 x 10^6 cells/ml	2 weeks	/	8 weeks	FT, B, BM	[208]
pig BM-MSCs	RGD-gamma Alginate/PEGMA/GelMA + PCL	20 x 10^6 cells/ml	4 weeks	/	6 weeks	FT, B, BM	[27]
Rat calvarial osteoblasts and bovine AC	RGD-modified alginate	39.4 x 10^6 cells/ml	/	/	4, 13, 26 weeks	C, B, BM	[209]
Death-Inducible hMSCs	Type I collagen mesh	70 × 10^6 cells/ml	3 weeks	2 weeks	12 weeks	C, B, BM	[210]
hBM-MSCs	Type I collagen mesh	40 x 10^6 cells/ml	3 weeks	2 weeks	5, 12 weeks	FT, B, BM	[211]
hBM-MSCs	Type I collagen mesh	70 × 10^6 cells/ml	3 weeks	2 weeks	5, 12 weeks	FT, B, BM	[170]
hASCs	Type I collagen mesh	40 x 10^6 cells/ml	4 weeks	2 weeks	12 weeks	B, BM	[212]
hBM-MSCs	Type I collagen mesh	35 x 10^6 cells/ml	3 weeks	2 weeks	4, 8 weeks	B, BM	[213]

characterization of native tissue development to identify quality controls suitable for a manufacturing process.

3. Go *In silico*

To understand the *in vivo* biological benchmark and assess the *in vitro* process for its ability to recapitulate said benchmark, *in silico* tools can be used. *In silico* refers to the use of computer models, in analogy to *in vitro* (in glass) and *in vivo* (in living subjects). *In silico* tools provide a way to quantify the biology at different time and length scales, ranging from the smallest metabolite in an individual cell, up to an integrated view on organ-level emergent behavior. Even though advances have been made on many *in silico* fronts, this section will focus on the progress and application of (single-cell) transcriptomics, metabolomics and systems biology approaches and their use in the context of DE (Fig. 3).

Table 2
Cell-based Tissue Engineered constructs for endochondral healing of orthotopic defects. Qualitative *in vivo* output was defined at the final time point with presence or not of S: scaffold remnants, TF: fibrotic tissue, C: cartilage, B: bone or BM: bone marrow. hBM-MSCs: human bone marrow stromal/stem cells, hUCB-BFs: human umbilical cord blood-borne fibroblasts, h/mPDCs: human/mouse periosteum-derived cells, hASCs: human adipose-derived stem cells, CHyA: collagen hyaluronic acid, PLGA: Poly Lactic-co-Glycolic Acid, N/A: not available/applicable.

Animal model	Cells	Culture set-up/Biomaterial	Cell density	Chondrogenic culture	Hypertrophic/osteogenic culture	*In vivo*	*In vivo* outcome	Total bridging	Reference
tibia 4 mm, mouse	hPDCs	Spheroids	250 cells/spheroid, ca 6000 spheroids/construct	3 weeks	/	8 weeks	B, BM	Yes (3/4)	[12]
tibia 4 mm, mouse	hPDCs	Spheroids, collagen type I hydrogel	250 cells/spheroids, 1200 spheroids/construct	6 days priming + 6 days	/	2, 4, 8 weeks	B, BM	Yes (5/6; week 4)	[138]
tibia 2 mm, mouse	hBM-MSCs	Pellet	2 x 10^5 cells/pellet	3 weeks	/	4 weeks	C, B	N/A	[214]
tibia 5mm, mouse	mPDCs	Collagen type I hydrogel	1 x 10^6 cells/scaffold		/	8 weeks	S, B, BM	Yes	[215]
lunate excision, rabbit	rabbit BM-MSCs	Esterified hyaluronan, gelatin	4 x 10^6 cells/ml	3 weeks	/	6, 12 weeks	C, B	N/A	[216]
femur, 7 mm, rat	rat BM-MSCs	Alginate	20 x 10^6 cells/ml	4 weeks	3 weeks	4, 8 weeks	S, FT, B	N/A	[217]
femur 8 mm, rat	h & rat BM-MSCs	RGD-functionalized alginate hydrogel	500 cells/spheroid 16h; 0.5 and 2.0 x10^6 cells/150 μL		/	4, 8, 12 weeks	S, B	No	[218]
femur 8 mm, rat	hBM-MSCs	Transwell + BMP-2 loaded hydroxyapatite microparticles + TGF-β1 loaded gelatin microspheres	3 x 2.0x10^6 cells/sheet	2 days	/	4, 8, 12 weeks	C, B, BM	Yes (8/9)	[13]
femur 8 mm, rat	hBM-MSCs	Transwell + TGF-β1 loaded gelatin microspheres	2 x 10^6 cells/sheet	2 days	/	4, 8, 12 weeks	C, B, BM	Yes (6/8)	[14]
femur 6 mm, rat	hBM-MSCs	Pellet	2 x 10^5 cells/pellet	3 weeks	/	4, 8 weeks	C, B	Yes (1 of 3 donors)	[219]
femur 5 mm, rat	hASC	Bovine decellularized trabecular bone	30 x 10^6 cells/ml	2 weeks	3 weeks	3, 6, 9 weeks	S, FT, B, BM	Yes (7/8)	[17]
femur 5 mm, rat	rat BM-MSCs	CHyA	1 x 10^6 cells/scaffold	3 weeks	2 weeks	4 weeks	S, B	N/A	[220]
femur 5 mm, rat	rat BM-MSCs	GelMA	20 x 10^6 cells/ml	2 weeks at 5% pO2 + 2 weeks at 20% pO2		2, 4, 6, 8 weeks	S, B, BM	Yes	[28]
femur 5 and 15 mm, rat	rat BM-MSCs	PLGA	3.15 x 10^6 cells/scaffold	3 weeks	/	4, 8, 16 weeks	S, B, BM	Yes	[221]
femur 2mm, mouse	mBM-MSCs	Demineralized bone matrix	N/A	2 weeks	2 weeks	2, 8 weeks	B, C, FT	Yes	[222]
cranial 8 mm, rat	mESC	Calcium phosphate ceramic	5 x 10^5 cells/scaffold	3 weeks	/	3 weeks	S, FT, B	n/a	[199]
cranial 7 mm, rat	rat BM-MSCs	CHyA and CHA scaffolds	1 x 10^6 cells/scaffold	3 weeks	2 weeks	4, 8 weeks	S, FT, B	n/a	[223]
cranial 7 mm, rat	-	Decellularised growth plate	/	/	/	4, 8 weeks	S, FT, B	n/a	[224]
cranial 5 mm, rat	hBM-MSCs	Transwell + BMP-2 loaded hydroxyapatite microparticles + TGF-β1 loaded gelatin microspheres	3 x 2 x 10^6 cells/sheet	2 days	/	4, 8 weeks	B, BM	n/a	[225]
cranial 4mm, mouse	hBM-MSCs	PCL micro-fiber scaffold	2.5 x 10^5 cells/scaffold	3 weeks	/	8 weeks	B, FT	n/a	[185]
cranial 3.5, mouse	hBM-MSCs	PLGA + hyaluronic acid-fibrin scaffold	5 x 10^5 cells/scaffold	1 week	/	4, 8 weeks	S, B	n/a	[226]

3.1. Transcriptomics

Over the past decade, the field of transcriptomics has rapidly evolved and redefined molecular and cellular biology, as well as TE. Bulk and single-cell RNA sequencing (scRNA-seq) technologies saw drastic advancements in their scalability and accessibility, and underwent a dramatic cost-reduction (reviewed in [35] and [36]). As a result, transcriptomes of tissues and single cells are now routinely sequenced [37] and full organism single-cell transcriptome atlases are being generated at a rapid pace [38–43]. Concomitantly, the engineering of DE implants/tissues from primary or induced pluripotent stem cells (iPSCs) is moving forward with great strides. The coming of age of the RNA sequencing technology allows its use for insightful analysis of the quality attributes of living engineered implants [44]. DE implants can now be quantitatively compared to their natural counterpart (i.e. corresponding developmental cascade) using transcriptome atlases as a reference for the quality of the DE implant. Moreover, these atlases can be studied to improve the differentiation protocols by making the growth factor cocktails resemble natural development more closely. Finally, by reconstructing developmental trajectories, it is now possible to determine the differentiation stage of cells and the distance to their arrival at a differentiated state that will result in functional outcomes, by taking a path similar to their *in vivo* (developmental) counterparts.

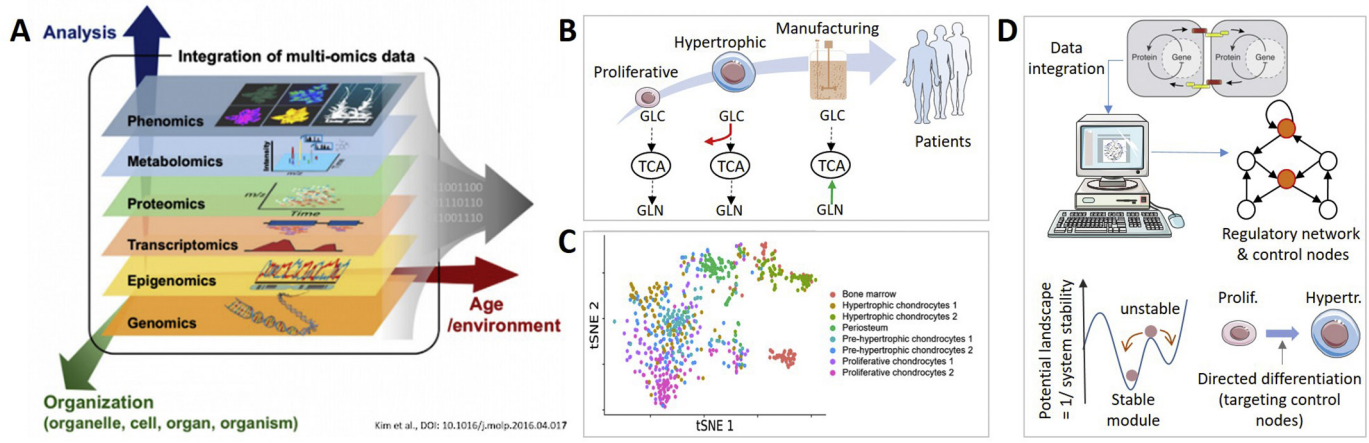

Fig. 3. Extracting DE relevant information using *in silico* tools. (A) From genes to phenotypes going through the omics layers. (B) Metabolic network states following the cascade of developmental processes events during endochondral ossification, providing the missing link between biomanufacturing and predictable outcomes. (C) Gene expression-based annotation of (single cell) transcriptomics data to generate an *in silico* reference of the tissue of interest. (D) Systemic integration of biological knowledge to build mathematical models such as regulatory networks. Identification of molecular targets to control cell fate and differentiation, e.g. from the proliferative (Prolif.) state to the hypertrophic one (Hypertr.), considering gene modules as stable phenotypes. GLC: glucose; GLN: glutamine; TCA: tricarboxylic acid cycle.

The similarity between the transcriptomes of natural and DE tissues can be described in terms of accuracy, precision and efficiency. In these cases, accuracy can be expressed as the fraction of the transcriptome that resembles the *in vivo* reference, and precision as the target to off-target ratio. Efficiency then translates to how well the engineered differentiation trajectory, *i.e.* the path taken by the cells to reach their final state, compares to development. These concepts are extensively reviewed in [45]. Nilsson Hall and colleagues successfully used transcriptomics as a quality control in their work describing developmentally engineered callus organoids [12]. In a time-series RNA-seq experiment, the authors showed that the temporal gene expression profile of the callus organoids resembles the formation of the growth plate in embryonic development and the soft callus during fracture healing. By the end of the *in vitro* maturation process, the transcriptome correlated to a pre-hypertrophic cartilaginous niche favoring mineralization and active remodeling of the extracellular matrix. Furthermore, the dynamic activity of signaling pathways elucidated by clustering of highly variable genes confirmed that the self-assembly and subsequent differentiation of periosteum-derived cells into callus organoids was an *in vitro* recapitulation of endochondral ossification. Bulk RNA-sequencing is informative on how well gene transcription compares on average with a given reference and whether or not the target cells have been generated, but has a low sensitivity for contaminating off-target cells.

While bulk RNA-seq captures the average transcriptomic profile, scRNA-seq captures unbiased snapshots of heterogeneity within the culture or tissue. It will be particularly interesting in the future to see how cell subpopulations within these organoids organize and guide the ossification process. Such analyses using single-cell transcriptomics have already been applied in a wide range of organoid models including kidney [46], retinal [47], neuronal [43,48–51], liver [52], pancreatic islet [53] and muscle organoids [54]. Importantly, these studies have demonstrated that differentiation protocols are robust and reproducible. Moreover, they have proven that scRNA-seq is capable of detecting contaminating cell types and time points at which gene expression deviates from natural development by activating off-target genetic programs. This in turn allows for a further refinement of the differentiation protocol. To our knowledge, no scRNA-seq datasets of skeletal organoids following a DE strategy as outlined in the previous section have been published as of yet, although a strong foundation has been laid. Recent work on the development of limbs in murine and chick models presents trajectories of lineage specification during embryonic limb development. This can serve as a useful resource for DE studies by dissecting subsets of the limb population related to bone development [38,55–57] and identifying central cell-cell communication networks [58–60]. These can be used in turn to more adequately tune growth factor supplementation of the differentiation media.

An important issue which needs to be addressed by DE methods is that cellular behavior is orchestrated by spatiotemporal signaling cues, organized in a specialized niche microenvironment [61], which most *in vitro* microenvironments fail to provide. The characterization of these factors by scRNA-seq enables the identification of stem cell nice-specific factors and the discovery of skeletal stem cells [62–64]. However, one major disadvantage of scRNA-seq in this regard is the requirement for tissue dissociation prior to sequencing. Methods to directly sequence single cells *in situ* are now being developed to address this issue [65–67] and have already been successfully applied to the bone marrow [68]. As spatial sequencing becomes more widely accessible, it will allow us to study intercellular crosstalk and provide novel insights in the regulation of morphogenesis, for which mathematical modeling can come to the aid as discussed in the next section. Capturing the small-scale regulatory mechanisms and feedback loops that underlie morphogenesis as seen in the developing growth plate will allow the evaluation of organoid patterning and engineering of niches by adding missing or previously unrecognized patterning signals to the culture strategy.

3.2. Systems biology

Since the availability of omics data is substantially increasing as outlined in the previous section, it has become clear that studying solely a couple of expressed genes would not be enough to describe tissue status or biological processes. Systems biology regards the ensemble of molecular mechanisms happening in a cell or a tissue as whole, in which all factors are interconnected and might influence the rest of the system. For instance, a systemic view of omics data already helped define regulatory mechanisms for cartilage cells phenotypes [69]. Moreover, systems biology is perfectly suited to assist tissue engineers who need precise design and control to follow developmental paths since it applies well-establish engineering principles from other industries and disciplines to the context of biological processes. Lenas *et al.* already formulated this need for applying engineering principles to DE a decade ago, emphasizing the importance of cell and tissue modularity, module robustness, tissue self-organization, cell fate and process controllability

[3,70]. Here, we review how mathematical modeling and network science can assist the DE process design and control by reviewing important achievements as summarized in Table 3.

DE requires a global understanding of signaling pathways involved in cell fate decision to identify the relevant cues leading a developmental trajectory in a specific direction. Interpreting those large molecular maps in mathematical terms, with quantitative or qualitative methods [71,72], makes it possible to evaluate the system's dynamics, thereby allowing *in silico* recapitulation of developmental events and prediction of the system's evolution under external cues. This has been frequently used in the context of stem cell engineering for regenerative medicine as previously reviewed [73]. Lenas *et al.* argued that the so-called macroscopic phenotypic modules, critical to the acquisition of proper TE constructs, result from the reachability and robustness of underlying gene/protein regulatory modules. On the one hand, reachability of a module can be evaluated by considering it as a mathematical system's stable state or attractor, comparable to a stable phenotype as formerly theorized [2,74,75] (Fig. 3D). Inputting initial conditions to the *in silico* system amounts to placing cells in a molecularly defined environment, activating transduction signals and eventually pushing the cells to acquire a new characteristic protein/genetic profile. Kerkhofs *et al.* showed that inputting random initial conditions to a regulatory model recapitulating chondrogenic differentiation in the growth plate resulted in the emergence of two biologically relevant stable states, resembling known proliferative and hypertrophic phenotypes of that cell type [76]. Such a mathematical representation allows *in silico* interventions for *a priori* identification of key molecular targets or driver conditions promoting the transition from one cellular state to another (e.g. transition from a proliferative chondrocyte to the hypertrophic state) [76–79] (Fig. 3D). Studying conditions that reach a specific state is valuable as it would enable the definition of a strict experimental 'priming', and, by extension, of a critical differentiation protocol [80,81].

Ideally, a minimal number of molecular targets (genes, proteins, transcription factors *etc.*) modulated in the regulatory network should be defined in order to finely control (stem) cell differentiation. When little kinetic and dynamic information is available, network science and control theory provide relevant tools to determine key control nodes from the structure/topology of the network alone (i.e. the nodes and ensemble edges between the nodes) (Fig. 3D). This was nicely theorized for biological systems by Barabási [82–84]. With such a method, Kobayashi *et al.* recently proved *in silico* and validated *in vitro* that simultaneous manipulation of 5 molecules among a network of 92 was sufficient to reproduce the cell specification of six different tissues in the ascidian embryo [85].

Another challenge that DE attempts to tackle is the morphogenetic and self-designed cell organization due to internal driving cues, so-called emergent behavior. Integrating information at one level of organization (intracellular, cellular) in order to answer questions at a higher level (e.g. macroscopic tissue structure, morphology) is explicitly facilitated by systems modeling approaches thanks to mechanistic coupling of subcellular signaling pathways to cellular behavior and macroscopic tissue organization [86–89]. Examples of applications are computer model-aided study of pattern formation or morphogenesis during embryonic development such as chondrogenesis in vertebrate limb, growth plate patterning dynamics due to growth factor gradients or the pattern of neuronal subtypes specification due the underlying transcription factor network [90–92]. Recently, the morphogenesis due to epithelial and mesenchymal cell differentiation during early embryogenesis was replicated *in silico* by coupling mechanics governing cell-cell interactions with intracellular signaling pathways [93]. This provides mechanistic explanations allowing hypothesis testing and bringing out the critical mechanisms that developmental tissue engineers have to ensure in order to replicate the wanted tissue structures.

Finally, engineering tissues requires quantitative metrics to evaluate how much the engineered cells resemble their native counterpart, in other words, a metric to ensure the process quality. As mentioned in the previous section, such a metric can be derived from transcriptomic data when combined with modeling tools. Cahan *et al.* have used the establishment of the target cells' gene regulatory network (GRN) for this metric. They developed a series of tools exploiting microarrays, bulk and single-cell RNA-seq data [94–97] to reconstruct characteristic GRNs of various target cell types (stem cells, cardiomyocytes, *etc.*) that are used as a training dataset for a machine learning classifier. These tools allow tissue engineers to input genetic data of their own construct, assess quantitatively to which extend the target GRN is established and propose candidate transcription factors to be manipulated to improve the conversion. It gave remarkably good results for conversion of fibroblasts to induced hepatocytes [97]. Osteochondral tissue-related cell types are currently still missing in the public training dataset, offering interesting perspectives of extension to use such tool for bone TE optimization and manufacturing process control.

3.3. Metabolomics

Besides the previously discussed advancements in transcriptomics and systems biology, other omics technologies have known equally impressive progress, including the field of metabolomics. Apart from providing cells with the required energy and the building blocks for the cellular processes, there is increasing evidence that metabolism plays an important role in defining cellular properties [98–100]. Metabolomics has been of such crucial importance for the advancement of insights in the cancer field, that cancer is now increasingly considered to be a metabolic disease. For regenerative processes, the systematic study of the impact of metabolism is only a recent phenomenon.

Despite the fact that metabolic research in the field of developmental biology and developmental engineering is still in its infancy, the first

Table 3
Applying engineering principles, through Systems Biology approaches, to aid process design in developmental tissue engineering.

	From gene module to phenotype, optimal conditions	Cell organization to tissue structure/pattern formation	Directed differentiation	Assessment of tissue constructs and intermediates
Engineering principle	robustness, stability, modularity, reachability, directed intervention	multi-scale emerging behavior, self-designed organization	controllability, directed minimal intervention	modularity, quality control, directed intervention
Systems biology/network science	dynamical regulatory networks, convergence to attractors and transitions	coupling subcellular signaling to multicellular behavior	scale free network topology & control theory	machine learning based GRN reconstruction and quantitative evaluation.
Application example	Regulatory network of chondrocyte differentiation, convergence to attractors as emergent phenotypes and controlled transition between modules.	Spatially extended gene networks: coupling regulatory network with mechanics/cell-cell interactions to reproduce epiboly in zebrafish during embryogenesis	Network structure based identification of 5 molecules (among 92) for which manipulation is sufficient to reproduce cell specification of six different tissues in the ascidian embryo.	CellNet: assess level of hepatocyte specific GRN establishment in induced hepatocytes and propose candidate TFs to manipulate for directing cell differentiation
Example reference	[76]	[93]	[85]	[97]

studies discussing the important role of metabolism in development are almost a century old [101]. In these early studies there were indications of spatiotemporal regulation of metabolism during development as metabolic features were shown to be tightly connected to developmental patterning [101,102]. With recent advances in mass spectrometry, a detailed description of metabolic signatures during development is now possible, resulting in a plethora of mechanistic studies into the role of metabolic changes as drivers of (patho)physiological processes. Taking bone regeneration as an example, metabolic alterations appear to be strong drivers of the regenerative process of defect healing [103] through their influence on cell differentiation and extracellular matrix production [104,105]. Metabolomics assays provide us with insightful information regarding the distinct metabolic features of the different cell types at different time points during the endochondral ossification process but also regarding the metabolic interplay between these cell types. For instance, MSCs in an undifferentiated state are mostly glycolytic mainly because of the hypoxic environment [106]. Differentiated chondrocytes also depend mostly on glycolysis and oxidative phosphorylation, which is generally decreased during chondrogenic differentiation [107]. Glucose, apart from being the cell's main energy source, appears to be crucial for the synthesis of extracellular matrix. Furthermore, metabolites have been shown to have signaling functions, either directly or indirectly. For example, during the bone fracture healing process lactate contributes to extracellular matrix synthesis and soft callus progression in an autocrine/paracrine manner [108]. Additionally, lactate accumulation (as a result of the glycolytic environment) induces the production of growth factors such as vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-b) and thus attracts MSCs and endothelial cells [109,110]. Moreover, recently it has been shown that chondrocyte metabolism controls the collagen synthesis and modification [104]. Interestingly, nutrient availability and more specifically extracellular lipid availability is an important determinant of chondrogenic commitment production [114].

As developmental engineering approaches have become increasingly successful over the last decade, there is also an increasing need for translation of laboratory scale experiments into industrial processes (discussed in the following section). Despite the recent advances in the field of developmental engineering, the biological complexity and the incomplete understanding of mechanisms of action as well as difficulties in product characterization have hampered the development of robust and standardized bioprocesses needed for the clinical translation. A thorough understanding of metabolism, as the intracellular level of regulation closest to the phenotype, might allow to identify critical quality attributes (CQA) which provide the missing link between the manufacturing practices and a predicable outcome [111,112] and allow to standardize the metabolic profile as a potency indicator [112]. Metabolomics data together with other omics data sets can be the starting point for systems modeling linking the metabolic network state with the desired phenotype (discussed above in the systems biology section) In that way, the next step would be metabolic modeling-based optimization of living implants production, which is already applied successfully in the biopharmaceutical industry [113].

While single cell techniques in transcriptomics are in rapid growth, single cell metabolomics is still in its infancy although a number of studies have demonstrated the feasibility of this technology [114–117]. In the coming years, with the experimental and computational advancements [118], single cell metabolomics integrated with single cell transcriptomics and proteomics, is expected to reveal important knowledge that is currently missing regarding the heterogeneity of developmental modules and their spatiotemporal regulation. This will provide crucial information on how the environment influences the developmental phenotype and will provide tools for quality control of developmentally inspired manufacturing processes.

4. Delivering DE implants – the path to the patient

With several DE-based *in vitro* strategies leading to solid biological results in small animal models (*ectopic and orthotopic*) and with *in silico* tools in hand allowing to precisely compare the engineered products to their developmental counterpart, as well as derive relevant process biomarkers, the field is now aiming towards upscaling and clinical translation. This upscaling requires the availability of sufficient quantities of cells able to robustly execute the biological processes and of the technologies to produce both the individual building blocks and the whole implant. In addition, a transition from small to large animal models and from manual laboratory protocols to GMP-compatible manufacturing processes able to pass regulatory scrutiny are important steps in the clinical translation process (Fig. 4).

4.1. Bone by design

Manufacturing capacity has been outlined as one of the major challenges to overcome in order to develop a sustainable healthcare sector [119]. For market success (and therefore clinical success), developmentally engineered constructs will need to be manufactured by cost effective, supervised, scalable and robust bioprocesses, meeting the requirements of regulatory bodies in terms of quality controls (QC) and good manufacturing practice (GMP). Manufacturing must be automated as even for current cell therapy, manufacturing processes rely on a great deal of time, manpower and cleanroom space, all of which can lead to a burdensome cost of goods (CoGs) with high overhead operating expenses associated with idle capacity [120]. One of the biggest challenges in cell and tissue manufacturing is to interconnect product quality attributes with their corresponding process conditions, something that will materialize through process automation. The ability to build proper quality profiles within living building blocks (such as DE constructs) that guarantee functional performance by operating the process in designed conditions, is referred to as the quality by design (QbD) paradigm [111,121]. The importance of the DE approaches is that their design and development is based on the existence of robust

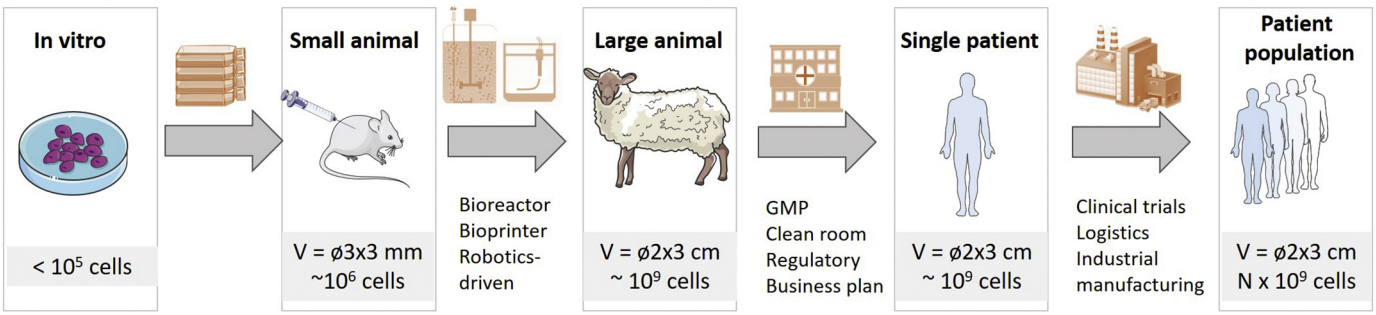

Fig. 4. Upscaling pipeline and stepwise increments from basic science to clinical uptake, indicating the important processes for each of the steps as well as a rough estimate of the volume (V) of the construct and the amount of cells needed to build it at each step. GMP: good manufacturing practices; N: amount of patients.

biological mechanisms upon which the function of the implant relies. This allows for the dissection of a profile of CQAs that could act as forecasting parameters of the performance of these living implants upon implantation. Although validated biomarkers that could constitute critical quality attributes are currently lacking, there are a number of potential candidates including expression of key genes such as collagen type X (ColX), Bone sialoprotein (iBSP), indian hedgehog (IHH) and metalloproteinases, which are reminiscent of events taking place at the growth plate transition zones [12,122]. Similarly, secreted molecules and proteins indicative of engineered cartilage maturity or related events should be identified. Additionally, the presence of adequately mature cartilaginous matrix can be investigated in situ as shown recently by the non-destructive technique of Raman Spectroscopy [123]. Through this technology, presence of minerals or mineralized matrix indicative of sufficient degree of differentiation can be detected and could be considered as a CQA. These measurable attributes could be monitored *in vitro* and allow active optimization of the cell expansion and tissue differentiation processes. The gradual process of moving from biopsies or frozen cell vials to the final implant of clinically relevant size is discussed below (Fig. 4).

4.1.1. Progenitor Cell expansion

Achieving the necessary scale and a sufficient quantity of progenitor cells is important for treating large defects such as those encountered in critical-size tibia defects (for example segmental defects larger than 3 cm). For such indications cell quantities ranging from 500 million to 1 billion will be needed [124]. Bioreactor systems allowing efficient expansion of progenitor cells should be used and evidence of this has already been provided for example in multi-stack and hollow-fiber bioreactors [125–127]. There exist several types of suspension culture using microcarriers in stirred bioreactors [128,129] such as vertical wheel systems [130,131], wave bags [132] and mostly small scale stirred systems (typically spinner flasks of 100-200 ml) [133–135]. Studies showing that BM-MSCs can be expanded in 5L stirred tanks [136] and 50L Mobius® bioreactors demonstrate that large scale production of single cell progenitor cells is feasible and could provide adequate quantities of cells for further differentiation. The use of microcarriers in stirred conditions could provide an advantage for the expansion of relevant cell populations to DE since upon controlled agglomeration of microcarriers, an environment of initial condensation can allow the priming towards the expansion of a more chondrogenic progenitor subpopulation [137], something that can be enhanced by using human platelet lysate [128].

A crucial aspect for manufacturing of improved DE implants would be the discovery of markers that allow for the separation and expansion of cell subpopulations with enhanced bone forming capacity while getting the necessary quality and quantity of cells. The recent discovery of markers that enable the identification [64], isolation and/or selective expansion (priming) [138–140] of potent skeletal stem cell populations is poised to revolutionize manufacturing strategies regarding cell-expansion. These candidate cell populations have been described recently, with special attention to those populations expressing a panel of cell surface markers indicative of a human skeletal progenitor population (PDPN+, CD146−, CD73+, CD164+) [64,122,140]. Those human progenitor cell populations displaying optimal characteristics for application in skeletal DE approaches could then be identified and isolated. Alternatively, it might be possible to derive these cell populations *in vitro*, as shown with murine iPSC-derived limb bud progenitors [141]. Noteworthy, preservation of the genomic, proteomic and regulatory landscapes (correct cellular identity) upon *in vitro* manipulation is key to proceed with DE approaches. Cellular identity greatly impacts processes of downstream cartilage intermediate tissue maturation and hence the ultimate potency of DE products.

4.1.2. Manufacturing of Cartilaginous Tissue Building Blocks

As described in section 2, functional DE-inspired cartilaginous building blocks can be developed in in a variety of ways (spheroids, sheets etc.), with *in vivo* bone formation capacity demonstrated in ectopically and orthotopically in small animal models. However, in order to evaluate their use in models that approach the human case, considerable upscaling is required to reach clinically relevant volumes of tissue building blocks.

Currently, a number of technical challenges still need to be addressed for future mass production of the tissue building blocks and only few studies demonstrate their scalable production. For the specific example of spheroids and microtissues, a number of commercial products allow manual generation of spheroids and scale-up of such platforms has been suggested [142]. Two main technologies will be discussed in more detail here, cartilaginous differentiation in (i) planar microwell systems and (ii) suspension culture in stirred tank systems.

Regarding the first option, proof of concept data has been demonstrated in microwells in numerous studies [143] for cartilaginous differentiation while the use of meshes has been suggested in order to address the typical issue of spheroid suspension and uncontrolled agglomeration [144]. However, given that millions to tens of millions of such spheroid/microtissue modules will need to be produced to reach clinically relevant implant volumes, corresponding to hundreds of microwell plates per implant, manual operation is deemed unfeasible. Robotics systems able to perform routine media changes could provide an alternative for the handling the required scale-out. Alternatively, suspension culture could be employed and several reports discuss the use of shaken [145] or stirred [146,147] systems, although spheroid aggregation is a challenge that should be tightly controlled in such systems to prevent the formation of mega-clusters with diameters exceeding millimeter size [148]. Computational fluid dynamics models can be used to calculate average shear rates that should be targeted in order to ensure adequate mixing for each individual aggregate [149]. However, most of these studies work with aggregates and spheroids in an undifferentiated state, providing proof of concept that suspension of such initial cell clusters is feasible. There is still a lack of dedicated studies investigating the production of differentiated (chondrogenic) microtissues in suspension systems for use in a DE setting.

Scalable production of clinically relevant amounts of building blocks is currently a major challenge and bottleneck for the clinical implementation of certain DE strategies. The transition to bioreactor systems, where millions of building blocks can be generated, could aid in the development of fully automated bioprocesses and enhance overall production capability and throughput.

4.1.3. Manufacturing of whole Implants

For the production of the final tissue product (DE-implant), biofabrication technologies should be developed that allow the use of aforementioned tissue modules with or without the presence of scaffolds [150]. Taking again the example of spheroids/microtissues, bioprinting of these building blocks exhibits distinct differences from single cell bioprinting and poses challenges in terms in terms of homogeneity of spheroid distribution in the resulting macro-tissue. Differentiated cartilaginous microtissues have recently been bioprinted in GelMA bioinks [151]. Despite some indications of positive viability readouts [152] as compared to single cell suspensions [152], there is a lack of dedicated studies on bioprinting process parameters and their impact on viability of the printed spheroids. A wide range of bioinks with suitable rheological behavior has been developed for extrusion bioprinting, mostly designed however for single cell suspensions. Therefore, new specifications should be pursued, such as the capacity of bioinks to remodel and to enable cell condensation and the formation of dense cartilaginous structures [153].

For the scaffold-free approaches, microtissues need to be positioned and allowed to assemble through a controlled process. In a novel scaffold-free bioprinting approach, modular cartilage tissue strands, fabricated by fusing tissue spheroids in a confining mold, were capable of being printed into 3D constructs using a robotic dispensing system

[154]. Recently, aspiration-assisted bioprinting showed that single spheroids (diameter below 1 mm), could be picked up and transferred to desired locations, showing an enhancement in precision in the positioning of such spheroids in the order of approximately 100 μm [155]. However, the throughput of these approaches is still low and considerable time (tens of hours) would be needed for the biomanufacturing of clinically relevant tissue volumes, possibly compromising biological functionality. It is plausible that through parallelization and further intensification of these processes fully automated production of modular skeletal tissue implants could be achieved in the future. Another approach using condensations of organoid structures was termed 'sacrificial writing into functional tissue (SWIFT)' and illustrated that it could be possible to rapidly condense (within minutes) suspensions of organoids into a viscous 'slurry' that allows self-assembly of organoids and produces organoid-based modular structures, thus addressing the manufacturing time challenge - albeit compromising the aspect of control [156]. The use of large spheroids for bioprinting has been exhibited [157,158] also for the formation of spatially organized tissues [159] in the presence of concomitantly bioprinted scaffolds and electrospun meshes [160]. For the biofabrication of larger tissues with increased complexity, bio-assembly and bioprinting technologies or the combination of these should be employed. This is a growing field of interest, requiring the merger of multiple biofabrication technologies resulting in hybrid strategies. For a review of this trend, see Dalton et al. (2020) [161].

A major aspect that is currently lacking in the aforementioned technologies is the capacity to implement quality controls in relation to the building blocks selected and used for the upscaling of the implants. Image-guided bioprinting strategies able to select and process quality-certified building blocks are under development and will enable the implementation of true "Bone by Design" strategies.

4.2. Size does matter

The development of clinically relevant DE-inspired constructs *in vitro* requires the use of "proxy" systems displaying decreased complexity and/or size when compared to their native developmental counterparts. These small-scaled TE constructs remain indisputably useful tools for deciphering mechanisms of cell growth and differentiation in function of time and scaffold's architecture, as well as for evaluating the capacity to form tissues (ectopically and orthotopically) and integrate into the orthotopic environment in small animal models (see Table 1 and 2). Increasing the size of promising DE constructs while maintaining their biological response *in vivo* remains a complex step in the translational process from bench-to-bedside. A major hurdle in this process is the eventual dysregulation of otherwise efficient biological processes observed at small scales. Owing to the modularity and mechanistic basis of the DE strategy, it is hypothesized that upscaling will have less impact on the biological activity of the construct, providing mass transport and mechanical fitness are sufficiently addressed as discussed next.

4.2.1. The mass transport challenge

Building blocks such as spheroids used for differentiation processes have sizes that typically range between 100-300 μm in diameter. This size range prevents the build-up of detrimental diffusion-related gradients. Two unwanted outcomes can be linked to diffusion limitations: (i) increased cell apoptosis due to lack of nutrients or oxygen and (ii) inadequate cell differentiation or presence of ECM due to local lack of stimulating growth factors [162,163]. It is important to note that these dimensions are highly transient since the properties of differentiating cells and microtissues, may vary as the differentiation process proceeds. In large bone marrow derived stromal cell (BM-MSC) spheroids, containing 60 000 cells, no hypoxic conditions were seen in the center while a hypoxic core was detected in spheroids containing at least 250 000 MSCs [164]. Regarding diffusion of growth factors, more complex mechanisms are at play including active binding by cells through specific receptors and tissue-dependent diffusion properties [165]. For BM-MSC-derived cartilaginous tissues, such inhomogeneities have been encountered in pellets and micromasses [12,21,24]. By the existing literature, a maximum cartilaginous tissue diameter of 1000 μm could be reached without compromising *in vitro* quality characteristics such as homogenous positivity for cartilaginous ECM markers [162]. Low oxygen concentration is a potent promoter of chondrogenic differentiation of MSCs and chondrocytes display a higher tolerance to low oxygen concentrations as compared to different cell types [166–168]. However, large volume constructs, particularly those >1.5 mm in thickness, will display features of hypoxic conditions leading to poorer biological performance [169]. Homogeneous differentiation is of importance since it has been seen that domains that are not positive of cartilaginous markers do not participate in the bone forming process leading to the presence of fibrous tissue structures [12,170].

Large cartilaginous constructs (Ø 10 mm × 6 mm) cultured in flow-through perfusion bioreactors in channeled alginate hydrogels demonstrate the capacity to obtain homogenously differentiated large structures [159]. Moreover, to overcome increasing mass transport limitations in larger constructs, efforts have been made to design and develop advanced oxygen-generating materials [171], multi-chamber bioreactor configurations [172] and 3D biofabrication techniques (e.g. printing in suspension baths or fused filament fabrication 3D printing) to provide optimal diffusion capabilities through tailored permeability and prevascularization strategies [173,174]. An alternative way to functionally vascularize TE constructs is using the patient's own body as a bioreactor, where a large construct could first be anastomosed and biologically activated in a heterotopic location, using a superficial artery and vein before being transplanted into the defect. [175–178]. Additionally, to reduce the weight of upscaling/diffusion limitation pressures in DE strategies, cells could be pharmacologically or genetically manipulated to sustain their viability in the center of the construct, mainly through manipulation of HIF-1a and its metabolic-related pathways [179]. While the use of genetically modified or pharmacological manipulated cells may raise additional safety concerns, e.g. off-target effects, their clinical translation seems feasible. Indeed, both the FDA and the European Committee for Medicinal Products have given marketing approval to Plerixafor (a small molecule for mobilization of HSC) or ZOLGENSMA® (gene therapy for spinal muscular atrophy) for human use.

The incorporation of these strategies in integrated manufacturing platforms will lead to the ultimate biofabrication production plant for complex DE tissues of clinically relevant sizes, as suggested recently in groundbreaking works [169,180].

4.2.2. From small to large animals

Large-size animal models are an indispensable step towards clinical translation. Besides being useful for testing biological potency and integration of up-scaled implants, large animal experiments allow fine-tuning technical handling of the implant itself and testing the overall feasibility of the related surgical procedures (an often under-valued but crucial element of large animal models). For the skeletal TE field, the most relevant animal models are the goat, sheep and minipig [181–184] (Table 4). While orthotopic testing of DE approaches in small animal models has been performed extensively (Table 2) [12,185], the application of DE strategies in large animal models is lagging behind. Although some of the most relevant studies in large animal models follow the trend of stimulating endochondral bone formation by using BMPs or materials that promote the EO route (Table 4), these approaches do not conceptually fit in the DE paradigm. Indeed, these strategies depend on the availability and activity of BMPs and/or material(s), not on autonomous developmental processes that could be controlled and monitored before implantation.

Current research using large animal models for the repair of critical-sized bone defects shows a concomitant advantage of combining classical TE-constructs with progenitor cells and growth factors [187–189] (Table 4). However, only a fraction of these studies achieved full bridging of the defects, and only two reported full bridging in all animals

Table 4
Limited summary of reported critical-size segmental defect models for bone Tissue Engineering construct testing. TCP: tricalcium phosphate, PCL: polycaprolactone, HA: hyaluronic acid, PLLA: Poly L Lactic Acid. Ti: Titanium, ABG: autologous bone graft, GF: growth factor.

Animal model	Location and size	TE Construct	Manufacturing Technology	Strategy/Experimental groups	Results	Evidence of EO	Total Bridging	Reference
Sheep	Femur, 5cm	TCP	N/A	TE construct vs. autografts	significant bone in both groups	No evidence	Yes (frequency not mentioned)	[191]
Goat	Tibia, 3cm	β-TCP + autologous BM-MSC	?	dynamic vs. static cultured cells	dynamic culture enhances bone formation	No evidence	Yes (in scaffold + dynamic cultured cells - frequency not mentioned)	[227]
Sheep	Tibia, 3cm	PCL + β-TCP microparticles (80:20)	fused deposition modeling	Cortical bone vs. scaffold	Geometry of the scaffold guides bone formation	No evidence	NO	[228]
Sheep	tibia, 3cm	PCL-TCP + BMP7 + MSCs	fused deposition modeling	with/without BMP7 with/without cells	combination product (cells + GF) gives good regeneration	Yes (BMP7 group)	Yes (8/8 in ABG and Scaffold+BMP7 groups)	[187]
Sheep	tibia, 3cm	PCL–TCP + MSCs	fused deposition modeling	autologous vs allogeneic cells ABG as control.	bone regeneration similar in all groups	No evidence	No	[229]
Goat	Tibia, 4.2cm	PCL + gBM-MSCs	melt electrospinning & fiber-bonding	construct precultured *in vitro* (14 days) static or dynamic	dynamic culture enhances bone formation	No evidence	No	[230]
Sheep	Tibia,	cortical allografts + BM-MSCs	N/A	with/without cells	cellularized allografts lead to faster bone formation	Yes (Cartilage remnants)	Yes (4/4)	[189]
Sheep	Tibia, 3cm	PCL + Col1/CS	embroidery	only scaffolds	complete vascularization of scaffold	Yes (Cartilage remnants)	Yes (2/4)	[231]
Sheep	Tibia, 3cm	PCL-HA + BM MSCs	fused deposition modeling	scaffold implantation with/without injection of cells at 4w PO	Delayed injection of cells improves outcome	No evidence	Yes (8/8 in autograft and allogenic BMSC groups)	[190]
Pig	Tibia, 3cm	Allograft + ASCs + rhBMP2	N/A	allografts with/without cells + BMP2	addition of cells + BMP2 improves outcome	No evidence (experimental group)	Yes (3/3 in best group - Allograft + MSCs + BMP2	[188]
Sheep	tibia, 3cm	Ca3ZrSi2O9 + PCL	polymer sponge method	with/without PCL coating	bone regeneration in all groups	No evidence	No (partial bridging 12/15)	[232]
Sheep	Tibia, 3 cm	PC + PLGA-particles with VEGF/PDGF & BMP2	melt extrusion	with/without GF loaded particles	bone regeneration in GF groups, no additional effect of VEGF/PDGF/BMP2 vs BMP2 alone	No evidence	Yes (4/5 in best group - scaffold + BMP2)	[233]
Sheep	Tibia, 4,5cm	empty defect	N/A	mature vs immature animals, fresh defect vs non-union	Partial filling (up to 80%) in immature animals	No evidence	NO	[183]
Sheep	Tibia, 3,5 cm	PLLA:PCL + BM-MSCs	solution blending process	with/without scaffold with/without cells	No significant bone formation in any condition.	No evidence	NO	[186]
Sheep	Tibia, 3cm and 4.5cm	dicalciumphosphate Scaffold (CopiOs) + Periosteal cells + BMP6	N/A	fresh defect vs biologically exhausted defect	Better results in fresh defects	Yes (evidence of callus formation)	YES	[234]

(100%) without the use of allo/autografts [187,190]. Of note, different biological outcomes between small and large animal models have been reported owing to the different bone fixation methodologies employed [186]. Even in the successful cases, authors acknowledge several challenges such as a lower density of the newly formed bone, a variable degree of cortex reconstitution [191], a large variation in bone formation and a strong growth factor dependence for the bone formation process [187]. So far, experiments in large animals models have taught us the main causes for failure of current TE strategies: uncertainty about cell number and their state of differentiation, the time of transplantation, cell apoptosis, and growth factor immobilization on and release from the scaffolds and dosage [187]. As pointed out in this review, DE strategies hold the potential to overcome these limitations, which anticipates an exciting new decade for DE-inspired strategies. Studies performed in small animal models have set the stage for implementation of DE-relevant strategies in large animal models.

4.3. Regulatory aspects

The technological advances discussed in the previous section are culminating in several initiatives that aim to substantially reduce human interventions during cell and tissue manufacturing through robotics. Examples of such initiatives in the EU are the 'Autostem' project, aiming at integrating robotic and bioreactors for stem cell manufacturing (http://www.autostem2020.eu/), and the 'Tissue Factory' developed by Fraunhofer starting with cell extraction and incubation up to a fully automated factory approach for human skin equivalents (https://tissue-factory.com). For the skeletal tissue biomanufacturing domain the 'Jointpromise' project (http://www.jointpromise.eu/) foresees the manufacturing of affordable complex organoid-based implants through the use of robotics and bioprinting. In the US, the BioFabUSA platform (a public-private partnership between the US Department of Defense and the Advanced Regenerative Manufacturing Institute) focuses on the production of scalable GMP-compliant manufacturing processes incorporating advances in biofabrication, automation, robotics and analytical technologies. The development of such end-to-end automated platforms will enable precision, reproducibility and online measurement and follow-up of CQAs, hence facilitating the regulatory process. These ongoing programs illustrate that the capacity in the field is constantly growing and maturing towards the delivery of proof-of-concept data in scales that could be representatives of real clinical scenarios.

Additionally, strong translational platforms are needed to generate more than a proof of principle, and obtain feasibility, safety and efficacy data in animal models relevant to the envisioned patients. Clinical

studies are often poorly designed and underpowered, and inappropriate comparators are used. Solving this is crucial in de-risking future clinical implementation. We expect the adoption of DE strategies will considerably facilitate the navigation of the complex regulatory landscape [111]; this is mainly due to the strong biological mechanisms and associated process traits underlying the function of implants produced through DE strategies (cfr Table 3). A quality target profile that will be constituted of orthogonal assays, *i.e.* explaining the function of the implant via independent mechanisms, would enable the production of safe and efficacious products.

Standards exist for a number of the processes discussed in this review, such as the ISO/DIS 23033 standard for characterizing and testing cellular therapeutic products. Other standards are related to the enabling technologies used to generate regulatory evidence, such as the ASME V&V40 standard providing a framework to assess credibility of *in silico* models used in the development of medical products. Other standards are still under development such as those for a large number of biomanufacturing technologies. This constantly developing regulatory landscape for TE products creates a big challenge for individual labs, smaller consortia or small and mid-size enterprises (SMEs). An open discourse is needed on the relevant and required measures, evidence and arguments that will eventually provide convincing statements that each stakeholder can support. A flexible, yet well-structured plan should be defined early in the innovation process and in conjunction with regulatory bodies.

5. The future awaits

This review has provided an overview of the basic concepts of developmental engineering in general and its application to bone tissue engineering in particular. It has discussed the enabling technologies such as multi-omics analyses and systems biology approaches that can help to realize a biomimetic *in vitro* process. It also provided an overview of the upscaling and manufacturing challenges linked to clinical translation and how DE approaches can facilitate meeting these challenges. DE offers a robust paradigm of biological events that provide to tissue engineers, markers and readouts that can link quality attributes of an engineered implant to its final performance and potency upon implantation. This recapitulation of innate mechanisms of development that can be quantitatively measured *in vitro*, offers a clear advantage to suboptimal and qualitative differentiation protocols that were followed to date. For the chondro-osseous continuum, a panel of biomarkers governing the transition across tissue zones provides candidate markers for linking potency to measurable markers during differentiation cascades minimizing failures and thus eventually facilitating industrialization. The increase of precision and throughput evidenced recently in biofabrication technologies as well as the merger with organoids sets the stage for the production of robust living implants that can in the future become even more complex and of enhanced functionality.

In regards to the skeletal application, this review does not go beyond the development of tissue constructs for critical-size segmental and osteochondral defects. More challenging skeletal applications such as the design and development of a whole joint or even a whole bone including joint surfaces are only in their infancy. Application of the proposed DE strategy and implementation of the aforementioned enabling technologies in other tissue and organ systems is also ongoing [97] and reviewed in [192].

There is a plethora of innovative and proven technologies that can play a role in the success of DE approaches that have not been discussed in this review. These include (non-exhaustively and in random order of importance) the use of induced pluripotent stem cells as a cell source, the use of gene editing techniques such as CRISPR-Cas9 to induce specific mutations and lead the cell population in a specific direction. Finally, this review has not gone into details on arguably the most important omics, being the economics [193]. Although translation of lab findings to the market will require DE products to be manufactured in a cost-efficient manner rendering them accessible to patient populations. This was in order to maintain a concise scientific perspective. For any TE construct, developmentally inspired or otherwise, to be viable commercially, the economic aspects need to be considered early on in the process of technology development. Concepts such as 'design to cost' are hard to implement when the R&D pipeline has been completed until (and including) the preclinical testing in large animal models. However, these concepts possibly are the ones making the difference between economically unviable treatments that cannot make it past the hospital exemption/compassionate use and economically viable treatments that provide biologically successful implants to those in need. The 'getting down to business' is the next big transition the TE field is facing, facilitated by the implementation of developmental engineering strategies.

Taken together it seems that the basic science stage is set, enabling the design and build of skeletal tissues possessing properties ensuring predictive performance *in vivo*. By merging this with breakthroughs in *in silico*, biofabrication and biomanufacturing technologies, and via the adoption of high-quality quality controls, robust DE products will be produced in the next decade. This will facilitate approval by regulatory bodies while attracting investments by lowering risks associated with market-stage product failure, and hence will contribute the creation of a viable healthcare sector able to revolutionize regenerative medicine.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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