Reverse Engineering Development: Crosstalk Opportunities Between Developmental Biology and Tissue Engineering

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ABSTRACT: The fields of developmental biology and tissue engineering have been revolutionized in recent years by technological advancements, expanded understanding, and biomaterials design, leading to the emerging paradigm of "developmental" or "biomimetic" tissue engineering. While developmental biology and tissue engineering have long overlapping histories, the fields have largely diverged in recent years at the same time that crosstalk opportunities for mutual benefit are more salient than ever. In this perspective article, we will use musculoskeletal development and tissue engineering as a platform on which to discuss these emerging crosstalk opportunities and will present our opinions on the bright future of these overlapping spheres of influence. The multicellular programs that control musculoskeletal development are rapidly becoming clarified, represented by shifting paradigms in our understanding of cellular function, identity, and lineage specification during development. Simultaneously, advancements in bioartificial matrices that replicate the biochemical, microstructural, and mechanical properties of developing tissues present new tools and approaches for recapitulating development in tissue engineering. Here, we introduce concepts and experimental approaches in musculoskeletal developmental biology and biomaterials design and discuss applications in tissue engineering as well as opportunities for tissue engineering approaches to inform our understanding of fundamental biology. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 35:2356–2368, 2017.

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REVERSE ENGINEERING DEVELOPMENT

Reverse engineering is the practice of disassembling a product to understand how it was made and how it works, to enable replication and manufacture of a similar object. Here, we propose that tissue engineering and developmental biology provide complementary and mutually beneficial perspectives for reverse engineering of living tissues with the dual aim to expand our understanding of the mechanisms that underlie tissue development and to advance functional tissue engineering.

Viktor Hamburger (1900–2001), one of the most influential developmental biologists of the 20th century, once stated: "Our real teacher always has been and still is the embryo—who is, incidentally, the only teacher who is always right".[1] In similar spirit, the polymath and mathematical biologist, D'Arcy Thompson (1860–1948), stated as introduction to his seminal work, On Growth and Form[2]: "But of the construction and growth and working of the body, as of all else that is of the earth earthy, physical science is, in my humble opinion, our only teacher and guide." With the aim of uniting these consummate teachers—the physical sciences and the embryo—we here highlight mutual opportunities for advancement of both tissue engineering and developmental biology through enhanced crosstalk. We propose that the benefits of this crosstalk are bi-directional, with unique potential to transform our approach to tissue regeneration by understanding and recapitulating natural morphogenesis, as well as providing powerful quantitative tools to developmental biologists to monitor, study, and modulate development.

This article represents an extension of a workshop organized and presented at the 2017 meeting of the Orthopaedic Research Society, and will use the musculoskeletal system, and specifically the process of endochondral bone formation as a model system to discuss the emerging paradigm of developmental, or biomimetic, tissue engineering, and to further discuss the opportunities for crosstalk between the fields of developmental biology and tissue engineering. We intend that the principles discussed here will have application and utility independent of the cells and tissues of interest.

In both developmental biology and tissue engineering, new technological developments and achievements have opened the doors for new questions, new goals and unprecedented control in the hands of scientists and engineers. However, with the increasing complexity of the tools, concepts, and theoretical frameworks, crossing these boundaries has become increasingly difficult despite increased "interdisciplinarity". We believe that much will be gained by the emerging crosstalk between developmental biology and tissue engineering in the years to come.

DEVELOPMENTAL ENGINEERING

Though most of our tissues emerge from development with remarkable regenerative potential (including accelerated wound healing and even regenerative

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capacity of cardiac muscle),[3] this potential diminishes rapidly with age, resulting in disease and impaired healing. Some vertebrate systems are capable of postnatal regeneration, including urodeles such as newts and salamanders, which exhibit near-perfect limb regeneration,[4] and some lizards, which feature "imperfect" repair.[5,6] Recently, the first observed mammal to exhibit this regenerative autonomy (in skin regeneration), the African spiny mouse (*Acomys*), has been described.[7] Notably, in all of these autonomous regeneration cases, the regenerating tissue features reactivation of developmental programs, including dedifferentiation of what were once thought terminally differentiated cells to an embryonic-like phenotype.[8,9] Even "imperfect" regeneration of the lizard tail, which forms a cartilaginous tube rather than a vertebral tail, recapitulates molecular programs of developmental endochondral ossification.[6]

With the absence of autonomous regeneration in humans, the field of tissue engineering and regenerative medicine has emerged to employ biological engineering approaches to repair and regenerate damaged and diseased tissues.[10] To date, however, successful translation of tissue engineering strategies from the laboratory to the clinic has not met the high expectations of the field's early years. Historical approaches in tissue engineering have primarily sought to replicate the properties of the mature tissue to be replaced[11,12]; however, the recent emergence of the "developmental" or "biomimetic" engineering paradigm has the potential to change the way we think about tissue regeneration. This concept argues that those processes selected for the formation of tissues in development may be highly efficient and potent for regeneration of those tissues later in life.

To accomplish this, tissue engineers will require detailed understanding of the critical mechanisms that must be replicated, including the effector cells, the environmental conditions, and the signaling pathways. Next, they will require the ability to accurately control morphogen presentation, matrix organization, and mechanical cues; and finally, they will need the tools to verify that the developmental programs were accurately recapitulated. Below, we discuss these principles using bone development and tissue engineering as a prototype to highlight this feedback loop, illustrated in Figure 1.

We therefore propose that revealing fundamental insights into the mechanisms that underlie normal development will enable development of truly biomimetic tissue engineering strategies that recapitulate the developmental programs for postnatal regeneration.

BIOLOGY OF BONE DEVELOPMENT

Starting from 270 bones at birth, the adult human skeleton is composed of 206 bones, excluding sesamoid bones. Among them, 80 bones are in the axial skeleton and 126 in the appendicular skeleton. During development, environmental biomechanical forces play important roles in creating different shapes (long, short, flat, and irregular) of bone. In embryogenesis, while most tissues come from one single germ layer, bone is uniquely derived from two types of germ layers, ectoderm, and mesoderm. Most facial and skull bones are originated from neural crest cells that arise from the crest of the developing neural tube and migrate out of the ectodermal layer to the other parts of embryo. Other axial bones (vertebral column and ribs) and almost all appendicular bones (limbs and girdles) are originated from mesoderm, to be precise, paraxial mesoderm (somites), and lateral plate mesoderm, respectively.[13]

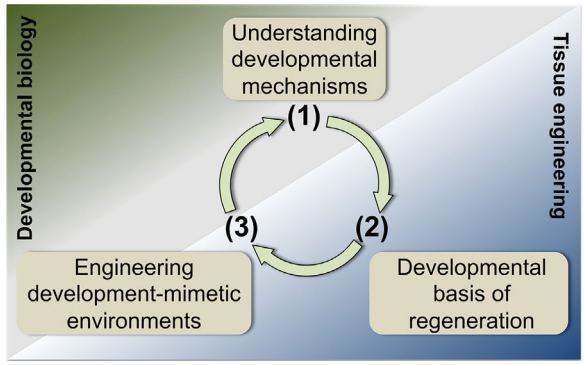

Figure 1. Proposed steps in mutual feedback between developmental biology and tissue engineering. Developmental biology insights inform regenerative approaches, enabled by engineered microenvironments, which in turn will enable novel approaches for hypothesis testing to understand developmental mechanisms.

Regardless of their origins, all bones are formed through two initially similar but later distinct mechanisms: Intramembranous and endochondral ossification. The former is responsible for the formation of most craniofacial bones as well as parts of clavicle and scapula, while the latter produces the majority of the axial and appendicular skeleton. Both mechanisms start with cell migration to the site of future bones followed by mesenchymal cell condensation. During condensation, rather than changing their proliferation ability, cells alter their adhesiveness to the extracellular matrix and to one another, migrate toward the center, and exclude vessels from the condensation. Eventually, the condensation reaches a critical size and a boundary is established to define the future skeletal element. Many genes, particularly those associated with cell adhesion, migration, and extracellular matrix, are critical for forming skeletogenic condensation.[14,15]

From this point forward, mesenchymal cells within the condensation adapt different fates depending on their expression of transcription factors. For intramembranous ossification, Runx2 and Osterix are the determinant factors that drive cells directly toward osteoblast differentiation for synthesizing type I collagen and other bone matrix proteins.[16] In contrast, for endochondral ossification, Sox9 is first highly

up-regulated in cells within the condensation core to initiate their chondrogenic differentiation.[17] When the cartilage anlage reaches a certain size, chondrocytes at the center stop proliferating and become hypertrophic. Meanwhile, mesenchymal cells at the condensation boundary begin to flatten, elongated, and form the perichondrium. Interestingly, the pre- and early hypertrophic chondrocytes in the cartilage anlage secret a cell signaling molecule, Indian hedgehog (Ihh), that directly stimulates their surrounding perichondrial cells to differentiate into osteoblast lineage cells, including osteoprogenitors and osteoblasts, and form the bone collar, a nascent form of cortical bone.[18] Later, osteoprogenitors in the perichondrium follow invading blood vessels into hypertrophic and calcified cartilage matrix in the center of anlage, and ultimately give rise to osteoblasts and osteocytes within the primary ossification center (POC).[19,20] Shortly after birth, while the POC continuously expands, canals originating from the perichondrium surrounding the epiphyseal cartilage begin to form and excavate into the cartilage center. These cartilage canals bring in blood vessels and mesenchymal progenitors to establish the secondary ossification center (SOC).[21,22] While the detailed signaling mechanisms are still largely unknown, it is clear that unlike bone formation at POC, cells within the perichondrium at SOC site do not undergo osteoblast differentiation, and the chondrocytes that the canals first penetrate are neither hypertrophic nor mineralized. The sequential development of the POC and SOC defines the location of the growth plate and articular cartilage in the long bone. Once the ossification centers are formed, both trabecular and cortical bones then undergo continuous remodeling, a process that starts by removing old/damaged bone matrix via osteoclasts and followed by depositing newly mineralized bone matrix via osteoblasts, throughout the entire lifetime. Past biology studies have demonstrated that each step of the skeletal development process is tightly controlled by multiple growth factors and transcription factors (summarized in Table 1). Interestingly, none of these growth factor-initiated signaling pathways is skeletal specific, indicating their essential and vital roles during the development of entire body.

These observations of developmental bone formation have several indications for designing new tissue engineering approaches for making bone in vitro or for in vivo regeneration. First, mesenchymal cell aggregation at a high cell density is critical for further skeletogenesis. Second, endothelial cells, the building blocks of blood vessels, should be first excluded from undifferentiated cell aggregates and then recruited back to the differentiated template. Third, to mimic endochondral ossification, a perichondrial layer of cells containing mesenchymal progenitors and endothelial cells should be considered to coat the cartilage rudiment for initiating bone formation. Last, various growth factors and transcription factors need to be embedded or expressed in the engineering constructs to spatiotemporally regulate the ossification process. Conversely, if tissue engineering approaches are sophisticated enough to reconstruct the skeletal tissues at various developmental stages using distinct populations of cells, scaffolds and growth factors, it would greatly advance our basic knowledge of molecular and cellular mechanisms in bone development.

APPROACHES FOR STUDYING BONE DEVELOPMENT

Genetically-modified mouse models have revolutionized our research on skeletal development by identifying proteins essential in this process and deciphering their mechanisms of action. A common way to manipulate gene expression is the Cre/loxP system in which a mouse carries both a transgene expressing Cre recombinase under a tissue specific promoter and a floxed target gene, namely, a gene with a region flanked by two loxP sites.[86] Cre can be further modified by fusing to a mutant estrogen receptor (ER) to ensure an inducible expression after Tamoxifen injections.[87] The commonly used promoters to drive Cre expression in bone development include limb bud mesenchyme-specific Prx1, cartilage-specific collagen type II (Col2a1) and aggrecan, hypertrophic cartilage-specific collagen type X (Col10a1),[88] osteoprogenitor-specific Osterix and αSMA,[20,89] mature osteoblast-specific Osteocalcin,[90] and osteocyte-specific Dmp1.[91] This Cre/loxP system can be designed not only for inactivation but also for overexpression of a particular gene. Fluorescent proteins with various colors represent a powerful tool to identify a particular cell type within a heterogeneous population of cells.[92] By inserting their genes at the endogenous Rosa26 locus downstream of a CAG promoter and a floxed STOP cassette, the expression of those fluorescent proteins serves as a faithful reporter for the Cre activity. Since Cre-induced recombination is irrevocable, all cells expressing the Cre activity and their descendants are labeled with the same fluorescent signal.

In the past several years, this lineage tracing approach has been used successfully to determine cell fate during bone development. For example, it has been debated over a century about where hypertrophic chondrocytes in the growth plate go during endochondral ossification. While the traditional view is prone to support a cell death fate when cartilage is transitioned to bone, recently studies based on lineage tracing using both non-inducible and inducible chondrocyte/hypertrophic chondrocyte-specific Cres as well as fluorescent reporters clearly reveal that at least some of those terminally differentiated chondrocytes could escape death and transdifferentiate into osteoblasts and osteocytes, thus directly contributing to bone formation.[93–96] Thus, lineage-tracing experiments provide important information about the cellular hierarchy that governs bone development and homeostasis. However, this approach is limited by the tissue or cell

Table 1. Major Growth and Transcription Factors that Govern Bone Development

Gene/Gene Product	Function	References
Growth factors		
BMPs	Establish the condensation size; promote both chondrogenesis and osteogenesis.	23–27
PTHrP	Secreted by perichondrial cells, PTHrP maintains proliferating chondrocytes and suppresses the onset of chondrocyte hypertrophy during endochondral ossification.	28–33
Ihh	Expressed by prehypertrophic chondrocytes, Ihh stimulates chondrocyte proliferation and is required for the synthesis of PTHrP. It also signals to the nearby perichondrial cells and directs them toward osteoblast differentiation.	32,34–38
FGFs	FGFs and their receptors are important for initiating mesenchymal condensation and its differentiation down the chondrogenic lineage. FGF-9 and -18 derived from perichondrium decrease chondrocyte proliferation and hypertrophy during endochondral ossification. FGFs also control all steps of osteoblastogenesis in a cell stage-dependent manner.	39–43
TGFβs	Initiate condensation formation; promote proliferation, chemotaxis, and early differentiation of osteoprogenitors but inhibit osteoblast maturation into osteocytes.	44–48
Wnts	Generally inhibit chondrocyte differentiation; potently stimulate osteoblast differentiation and bone formation.	49–56
Notch ligands	Attenuate mesenchymal condensation and subsequent chondrogenic differentiation; suppress osteoblast differentiation in mesenchymal progenitors.	57–61
VEGF	Released by hypertrophic chondrocytes, VEGF recruits blood vessel invasion into the cartilage matrix to initiate bone formation during endochondral ossification.	62–66
Transcription factors		
Sox9	Sox9 is essential for initiating chondrogenesis during endochondral ossification.	67–70
Runx2/ Cbfa-1	Runx2 is a master transcription factor for osteoblast differentiation in intramembranous and endochondral ossification. It also promotes the hypertrophic differentiation of chondrocytes.	71–77
Osterix/Sp7	As a Runx2 target gene, osterix is another essential transcription factor for osteoblast differentiation in intramembranous and endochondral ossification.	78–81
β-catenin	Primary effector of canonical wnt signaling; promotes osteogenesis; inhibits chondrogenesis.	82–85

BMP, bone morphogenetic protein; PTHrP, parathyroid hormone-related protein; Ihh, Indian hedgehog protein; FGF, fibroblast growth factor; TFGβ, transforming growth factor beta; VEGF, vascular endothelial growth factor; Sox9, SRY-Box 9; Runx2, runt-related transcription factor 2.

specificity of the promoter that drives the CRE expression and the mosaic expression of the reporter gene within the targeted cell population.

Immunohistochemistry (IHC) is another important approach that greatly advances our knowledge of skeletal development. Since it uses antibodies to semi-quantify the amount of proteins in a cell specific manner, IHC provides much more biological information in a heterogenetic tissue compared to real-time RT-PCR and Western blot that measures RNA and protein levels, respectively. This is particularly important when studying bone development as aforementioned, this developmental process requires multi-cellular interaction at any given step. Traditional IHC uses thin sections that only capture 2D information at one time point. Newly developed whole mount immunofluorescence combined with advanced confocal microscopy is advantageous for examining spatial information at an ultra-high resolution. It is particular useful for analyzing vascular network in bone because traditional thin sections lose the architectural information.[97] Moreover, real-time intravital fluorescence imaging,[98] which has already been used successfully in studying calvarial bone development[90] and regeneration,[99] should be a powerful tool to trace cell migration and differentiation during endochondral ossification when combined with the lineage tracing approach.

In addition to the above established approaches, other emerging techniques in the skeletal development field could also be adopted for tissue engineering studies. Those include, but not limited to, deep tissue clearing for whole mount examination,[100] laser capture microdissection for RNA and protein analysis,[101] and even more challenging, genome-wide profiling in single cells.[102]

THE NEED FOR REGENERATIVE APPROACHES

An estimated 126 million Americans are affected by musculoskeletal disorders and many of these patients could benefit from tissue-engineered cartilage, bone, and connective tissue constructs. Currently, developing cartilage constructs for integration and resurfacing joints, tendons for repair, and bone for treating large bone defects and for facilitating spinal fusion could be used to fulfill unmet clinical needs. However, while

inducing bone or cartilage lineage-specific differentiation in the laboratory is now common, production of mechanically and biologically functional tissues, or complex composite tissues that can be translated for use in patients remains challenging. By applying knowledge gleaned from studies on development of skeletal tissues, new approaches may be developed to generate translatable tissue constructs.

Of these skeletal tissues, bone exhibits a remarkable ability to regenerate after injury. The process of bone formation and regeneration is well-studied, and there are many potential avenues for translational research to have a sustained effect on the field of bone tissue engineering.

DEVELOPMENTAL BASIS OF REGENERATION

During embryonic development, bone forms by two distinct processes. Bones of the skull and the clavicle from by intramembranous ossification, a process in which precursor cells differentiate into osteoblasts and form bone directly. In contrast, during formation of the retroarticular process of the jaw, and the axial and appendicular skeleton, precursor cells differentiate into chondrocytes, which form a cartilage template that is replaced by bone through the process of endochondral ossification.

Both of these processes are recapitulated during bone healing. In mechanically stable environments stem cells located in the periosteum and endosteum differentiate directly into osteoblasts and the bone heals through intramembranous ossification.[89,103] In contrast, in mechanically unstable environments, stem cells in the periosteum differentiate into chondrocytes and the bone heals primarily through endochondral ossification,[89,103] with some direct bone formation within the endosteum and the periosteum at a distance from the fracture site (Fig. 2). Interestingly, the embryonic origin and history of the bone does not influence the mode of healing, but this is determined rather by the mechanical environment as even bones of intramembranous origin heal through the endochondral mode under conditions of interfragmentary motion.[104]

While the process of regeneration does indeed recapitulate bone development, there are significant differences between development and healing. After traumatic injury, there is an influx of all inflammatory cell types to the site of injury. These cells debride the wound, and stimulate healing. While there is no inflammatory response during bone development, tissue resident macrophages, osteomacs appear to be important during bone formation.[105] Additionally, endogenous mesenchymal stem cells are present at sites of injury, but their endogenous, in vivo functions remain unclear. For example, circulating progenitors have not been observed to give rise to regenerating cartilage and bone in a parabiosis model,[106] and native pericytes may not behave as stem cells in vivo,[107] despite clear multilineage capacity when cultured in vitro or implanted exogenously.[107–109] However, these cells may participate in and orchestrate the healing process, by providing signaling factors that help regulate repair.[110] Further research will be needed to elucidate the functions and capabilities of these cells, in development, homeostasis, and regeneration.

Developing novel constructs to treat fracture patients has been a long-standing goal in Orthopaedic research. Many investigators have developed bone grafts based on intramembranous ossification. Osteoblast differentiation is induced and a mineralized tissue constructs is allowed to form in vitro, then this construct would be implanted into a bone defect.[108,111,112] However, bone is highly vascularized and the tissue engineered constructs need to take this into account. Development of composite tissues can overcome this problem.[112–114]

We,[96,109,115,116] and others,[117–119] have proposed and demonstrated that cartilage grafts have the ability to heal large bone defects. The idea that cartilage could be used to heal bone is based directly on the fact that bone can form and heal fractures via endochondral ossification. Cartilage is avascular, but has angiogenic activity[120,121] so cartilage survives transplantation and induces the host vasculature to invade and convert the cartilage to bone.[96] Thus, by using developmental mechanisms as inspiration, novel therapies to treat bone defects can be designed.

FAILURE OF REGENERATION

An estimated 5–15% of bone fractures fail to heal in a timely manner.[122] Delayed healing or non-union creates significant health burdens and severely impacts the quality of life of affected individuals.[123] Too much motion at the fracture site leads to a hypertrophic non-union, in which, a large cartilage callus forms, but does not undergo endochondral ossification. This outcome requires a revision surgery to stabilize the fracture site, and healing usually proceeds normally. However, a number of other conditions, including diabetes, smoking, rheumatoid arthritis, and aging, are associated with poor healing outcomes possibly due to dysregulated inflammatory processes.[124] Further, concomitant vascular or nerve injuries are associated with delayed healing or non-union.[112,114,125,126] Therefore, developing therapies to target each of these patient populations could significantly improve fracture healing outcomes for a large number of individuals.

BIOMATERIALS FOR ENGINEERING DEVELOPMENT-MIMETIC MICROENVIRONMENTS

Expanding beyond the standard 2D culture on tissue culture plastic utilized in the cell and molecular biology communities for decades, new 3D systems have emerged which better replicate the cellular environment present during development, repair and homeostasis in the body.[127] These systems offer a powerful opportunity to regulate and study musculoskeletal cell behavior, and ultimately enhance our understanding of the critical signals needed to drive new tissue formation.

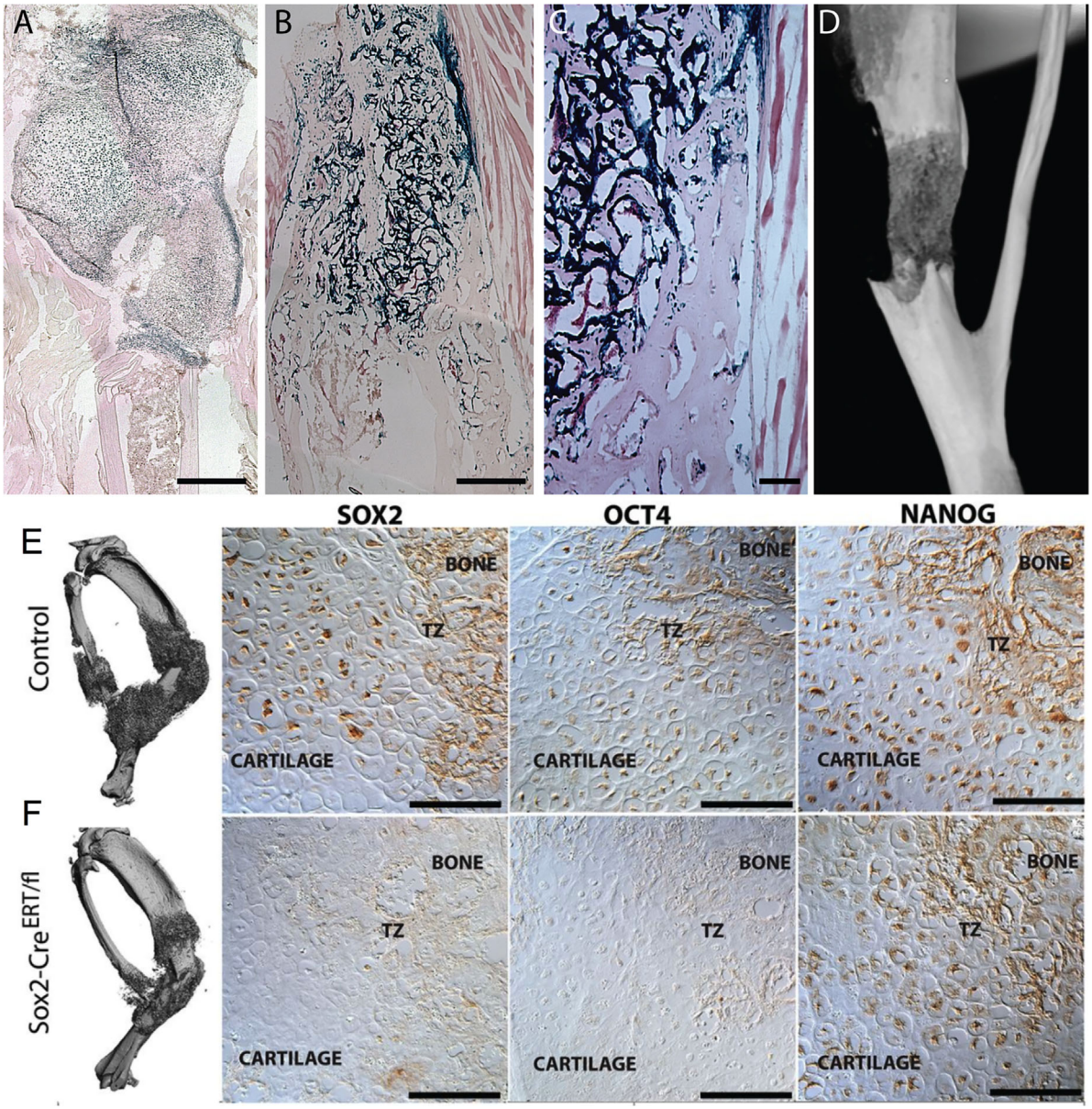

Figure 2. Transformation of Chondrocytes to Osteoblasts During Bone Fracture Healing. (A–D) Transplantation of cartilage stimulates repair of a segmental bone defect in mice. Cartilage was derived from ROSA26 mice that express the beta-galactosidase transgene ubiquitously, and donor cells can be distinguished from host cells by X-gal staining to label donor cells blue. (A) The cartilage graft at 1 week, and (B and C) 4 weeks after engraftment show that the newly formed bone is derived from the transplanted cells. (Reproduced with permission from JBMR. J Bone Miner Res. 2014; 29(5): 1269–1282). (E and F) Fracture healing in Wild type mice and after conditional inactivation of Sox2 using a $Sox2Cre^{ERt}$ deleter mice shows decreased callus formation and reduced Sox2 and Oct4 expression, and no effect on Nanog expression (reproduced From Development, 2017 144: 221–234). Scale bars A and B = 200 mm, C = 500 mm, E and F = 100 mm.

A primary approach to engineer musculoskeletal tissues involves using biomaterials as an architectural scaffolding that serve as a surrounding extracellular matrix for cell adhesion, proliferation, differentiation, migration, and/or communication with each other, a framework to provide mechanical support for tissue formation, and a mechanism for providing instructive signals to guide the function of seeded cells. These scaffolds can be comprised of biomaterials from natural sources (e.g., collagen, hyaluronic acid, alginate, chitosan, decellularized tissue), synthetic polymers or combinations of the two.[128–130] Their properties, such as biochemical composition, structure, mechanics, porosity, and degradation rate and mechanism, provide cues to cells and regulate their gene expression and behavior. Functionalizing synthetic polymers with natural materials provides advantages such as better control over the final product and enhanced mechanical properties inherent with synthetics, while permitting endowment with specific biological activity in a modular manner.

Soluble bioactive factors can be delivered from these scaffolds to guide cell fate as well. The factors can include growth factors, cytokines, transcription factors, hormones, and RNA inherent to developmental processes, in addition to other genetic material such as plasmid DNA that can program cells to produce proteins of interest. The biomaterials can be engineered to control the temporal presentation of one or more of these factors, with potentially different

profiles, by regulating, for example, their diffusion through the scaffold, their affinity to or interactions with the scaffold, and the scaffold degradation rate and/or mechanism.[131]

A more recent alternative strategy to the use of scaffolds in musculoskeletal tissue engineering involves partially recreating the high-cell density conformation of cells in immature mesenchymal condensations present during development.[132] Isolated stem cells in suspension can coalesce via cell–cell adhesion proteins into self-contained masses. When these cells are exposed to cytokines in culture media, they can be guided to differentiate into defined connective tissue phenotypes. Biomaterials microparticles can be introduced within these cell aggregate masses, and the microparticles themselves or delivered biologics can drive tissue-specific lineage progression.[133] Using this approach, tissues can be formed in a wide range of sizes and geometries, from spheres[134] to sheets[135] to rings and tubes.[136]

TOOLS FOR REPLICATING DEVELOPMENT

There is an extensive array of technologies and tools currently available that can facilitate the recapitulation of developmental microenvironments, and help identify conditions which could recreate them. It is well known that mechanical forces play a critical role during development. Bioreactors make it possible to control the mechanical environment of a growing cultured tissue construct, allowing the static or dynamic application of stresses, such as tension, compression, shear and/or hydrostatic,[137] which may be designed to mimic those present during development in terms of magnitude, frequency and duration. More recently, methods have been reported with the potential to modulate the mechanical environment in an actual tissue defect in vivo, permitting the role of this important signal on healing musculoskeletal tissues to be elucidated.[114] While there is currently incomplete understanding of the exact mechanical environment present during development, which in turns limits biomimetic approaches in this area, recent reports have begun to quantify these stimuli during development in both animals[138–140] and in humans.[141]

To understand the role of individual and combined signals that can influence cell behavior, such as those from biomaterials, soluble bioactive factors, mechanical signals and other cell populations, in an efficient, fast and cost effective manner, numerous high throughput screening systems have been developed.[142] These systems often utilize technologies such as microfluidics, microspotting, and/or microcontact printing. They have the capacity to screen hundreds to thousands of microenvironments simultaneously in a combinatorial manner, facilitating the understanding of how multiple signaling cues are interpreted by cells to elicit particular responses.

Tissues develop with precise spatial distributions of multiple cell phenotypes, extracellular matrix molecules and soluble bioactive factors. Recreation of some of these architectural relationships may be critical to harness the potential of biomimetic regenerative strategies, and 3D printing technologies facilitate the placement of these different tissue building blocks in defined locations with high resolution on the microscale.[143,144] Tools like 3D printing and microfluidics also support the formation of soluble signal gradients,[145] which are present throughout the development of musculoskeletal tissues. Using such tools, in conjunction with controlling the timing of, for example, biomaterial degradation or bioactive factor release, gives biologists and engineers the ability to truly recapitulate microenvironmental signals with temporospatial specificity.

APPLYING BIOMATERIALS AND ENGINEERING TOOLS TO RECREATE BONE DEVELOPMENT

Intramembranous ossification (IO) approaches to engineer bone typically involve seeding osteoblasts or osteoprogenitors onto or into a biomaterial scaffold, and then driving the direct formation of bone tissue through the controlled delivery of potent osteogenic soluble signals, such as bone morphogenetic proteins or genes encoding for these molecules. As mentioned earlier, recapitulating endochondral ossification by first forming a cartilaginous anlage that can then be remodeled and replaced by bone tissue may be a more advantageous route. This strategy has been pursued in several different ways, including incorporating both of the cells types critical for endochondral ossification (i.e., chondrocytes and osteoblasts) into a peptide modified hydrogel,[109] and delivery of chondrogenic and osteogenic signals to cells with controlled temporal profiles from biomaterials.[146] Interestingly, endochondral strategies may even be applied successfully to regenerate tissue where bone forms by intramembranous ossification during development.[146]

Enhancing angiogenesis is critical for the survival of cells in intramembranous approaches, especially where there has been substantial vascular injury, and for endochondral technology to bring in new vasculature along with progenitors cells capable of differentiating into osteoblasts and replacing engineered cartilage. Efforts in this area have focused predominantly on delivery cells capable of participating in or inducing angiogenesis (e.g., endothelial cells, endothelial progenitors, etc.), and controlled delivery of soluble factors that are angiogenic, that recruit vascular and supporting cells, and/or that help stabilize forming vasculature (e.g., VEGF, PDGF, SDF-1, etc).[115,147,148]

It is important that new development-mimetic tissue engineering approaches be assessed by standardized functional outcomes and appropriate benchmarks of success. New approaches must be validated first for their capacity to effect functional regeneration, including verification of restored mechanical function in comparison with intact, native controls and demonstration of mature tissue biological function, for example, endocrine activity and restoration of the hematopoietic niche. We envision that the development-mimetic tissue engineering paradigm will have particular promise in the area of pediatric tissue regeneration; therefore, it will be critical to achieve and quantitatively evaluate long term tissue growth and remodeling concomitant with patient ageing. Further, benchmarks of success for developmental engineering approaches should include comparison with the current state of the art and verification of developmental biomimicry. Thus, benchmarking will require head-to-head comparison with classical approaches and current clinical standards including scaffold and growth factor-based

techniques that may provide advantages in ease of clinical implementation. It will also require cell- and molecular-level assessment of the extent to which the engineering approach recapitulates the desired developmental process.

FEEDBACK FROM DEVELOPMENTAL TISSUE ENGINEERING TO DEVELOPMENTAL BIOLOGY

The recent re-emergence in the literature of "organoid" culture[151] has produced dramatic advancements in our understanding of stem cell and developmental biology for a variety of tissues from gut epithelium to the structures of the brain.[151,152] Notably absent in this modern revisiting of the organoid paradigm, which reached its former height in the 1960's–80's,[151] are the tissues of the musculoskeletal system. However, the principles of developmental engineering discussed here continue to gain traction in the musculoskeletal community,[96,118,119,146,153–158] and, with the recent and rapid expansion in biomaterial techniques available for controlling microenvironments, as discussed above, these principles are likely to contribute significantly to our understanding not only of how to engineer

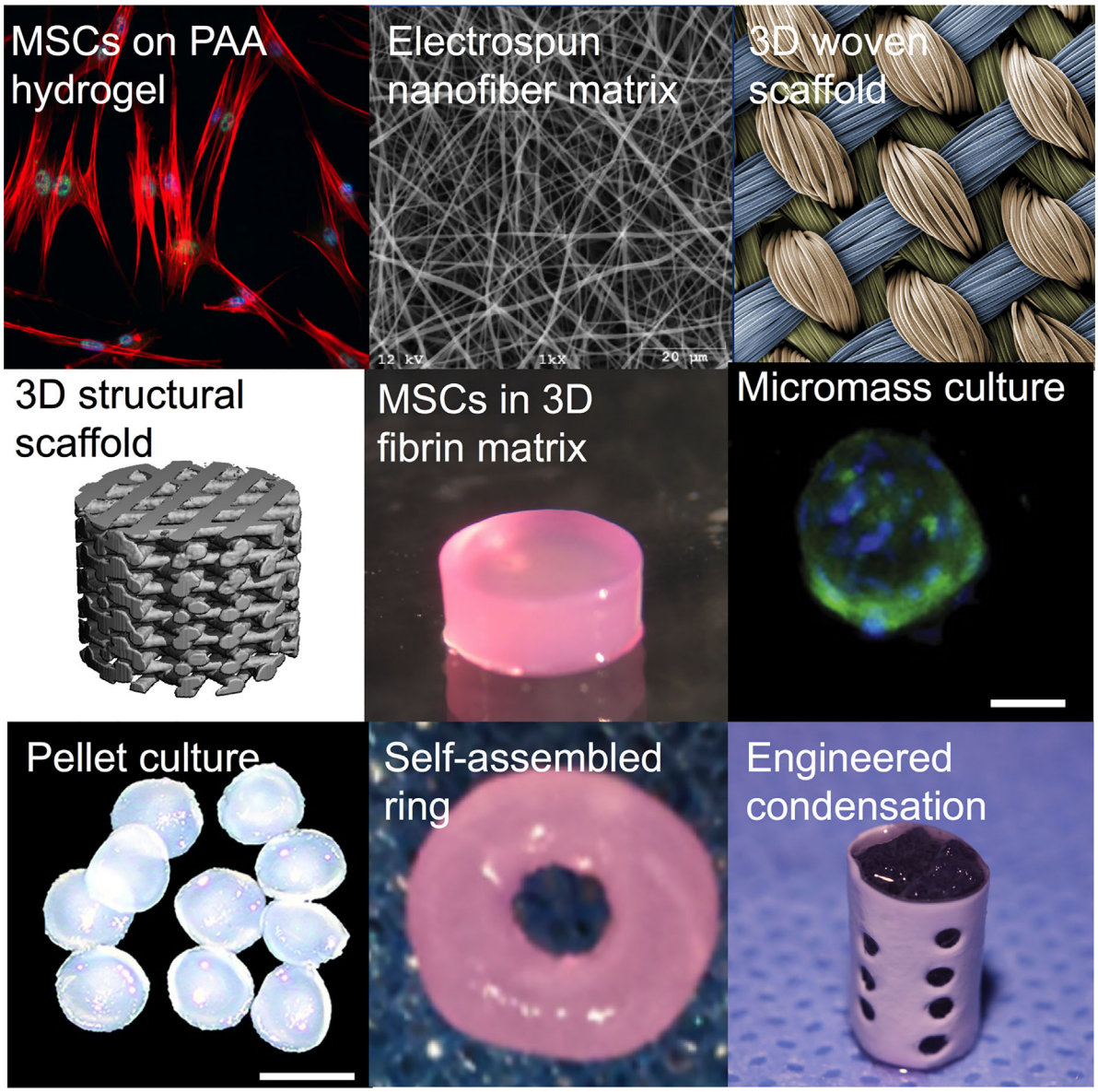

Figure 3. Biomaterial approaches for replicating developmental conditions in tissue engineering. Among emerging approaches include 2D cell culture on the surface of extracellular matrices engineered to mimic the biochemical or biophysical environment, such as functionalize polyacrylamide (top-left panel; Image credit: J. Boerckel) or electrospun nanofiber meshes (top-center; Image credit: Y. Kolambkar). Building in complexity, 3D matrices can be built up from woven fibers (top-right; Image modified from Moutos et al. PNAS 2016). Other 3D approaches include printed structural scaffolds (middle-left; Image credit: J. Boerckel), or hydrogel matrices enabling 3D cell distribution (center; Image credit: A. McDermott). Cellular assembly approaches that mimic the cell–cell interactions present in early limb development include micromass culture (middle-right; image source:[149]), pellet culture (bottom-left; image source:[150]), 3D cellular self-assembly (bottom-center; image source:[136]), and defect-filling engineered condensations (bottom-right; image credit: E. Alsberg, J. Boerckel).

functional musculoskeletal tissue replacements, but also to reveal the fundamental mechanisms underlying the natural development of these tissues.

Translational studies in rodents have and will continue to add to our understanding of musculoskeletal development. For example, transplantation of cartilage grafts into critical sized defects of murine tibiae uncovered that chondrocytes transform into osteoblasts during bone fracture healing.[96] Subsequent publications have confirmed this observation and shown that chondrocytes also transform into osteoblasts during endochondral ossification in the growth plate.[93–95,159,160] Similarly, engineering approaches[161] that explore the roles of mechanical forces in tissue formation and regeneration[114,162] are also capable of revealing important insights about the influence of mechanical cues in tissue morphogenesis and embryonic development.[163,164] Thus, tissue engineering advances health care by providing avenues to therapy and also by illuminating previously unknown developmental mechanisms.

UNANSWERED QUESTIONS AND FUTURE DIRECTION

Future work will continue to improve our understanding of the biology of development, including the spatial distribution and temporal appearance of the cellular actors, morphogens, and extracellular matrix molecules. Other areas for continued research and distinct need are improved techniques for both temporal and spatial control over the presentation of multiple soluble factors with different release profiles matched with optimal delivery vehicle biodegradation. Additive manufacturing techniques show promise for generating complex architectures with developmental inspiration,[165] and further research will be necessary to improve speed, bioactivity, structural integrity, spatial complexity, and compositional heterogeneity. Limits in vascularization for regeneration of large tissues remain a significant hurdle, and are likely to benefit substantially from observation of the mechanisms by which developing tissues accomplish this end.[112,114,158]

CONCLUSIONS AND RECOMMENDATIONS

We have presented a framework for synergistic advancement of our understanding of tissue development and approaches for mimicking this process for tissue engineering. With these considerations in mind, we make several recommendations for continued research in this area: First, the ultimate test of any regenerative approach must be functional regeneration, including restoration of both mechanical and biological function. Functional outcomes with comparison with native adult tissue beyond histological demonstration of tissue identity must become standard and requisite.[166] Second, as the field evaluates the efficacy of this emerging developmental approach, direct comparison with traditional tissue engineering approaches will be important to establish benchmarks for relative success in addition to ultimate tissue functionality. Third, regenerating tissues through developmental engineering approaches must be compared not only with the final mature tissue, but also to the developing tissues that they are intended to recapitulate to verify the accomplishment of the development-mimetic goal and to establish the full benefit of the feedback loop described in Figure 3. In addition to morphological and cellular composition, this will include quantitative comparison of the cellular and molecular mechanisms underlying both tissue regeneration and development.

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