ABSTRACT

Here, we review the use of human pluripotent stem cells for skeletal tissue engineering. A number of approaches have been used for generating cartilage and bone from both human embryonic stem cells and induced pluripotent stem cells. These range from protocols relying on intrinsic cell interactions and signals from co-cultured cells to those attempting to recapitulate the series of steps occurring during mammalian skeletal development. The importance of generating authentic tissues rather than just differentiated cells is emphasized and enabling technologies for doing this are reported. We also review the different methods for characterization of skeletal cells and constructs at the tissue and single-cell level, and indicate newer resources not yet fully utilized in this field. There have been many challenges in this research area but the technologies to overcome these are beginning to appear, often adopted from related fields. This makes it more likely that cost-effective and efficacious human pluripotent stem cell-engineered constructs may become available for skeletal repair in the near future.

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Introduction

Life means danger: accidents, injury, and disease are inevitable. Unfortunately, human beings do not regenerate such damaged tissues or organs as effectively as lower vertebrates. Amphibians and fish have greater regenerative capacity than mammals and can fully remake limbs, tails, and fins when damaged or lost [1,2]. Although our bones and livers will regenerate to a reasonable extent [3], most skeletal tissues such as articular cartilage instead resort to fibrosis and scar tissue formation in place of the original structure.

However, human scientific ingenuity has resulted in the development of novel tissue engineering (TE) approaches to aid correct cell replacement and generate authentic tissues and organs when our bodies fail to repair on their own. TE is the science of using external materials or constructs to stimulate the innate ability of cells to generate lost or damaged tissue of the right kind in situ, or to use multidisciplinary technologies to create those tissues from cells outside the body [4]. In both cases, the tools available to us are improving rapidly. However, the choice of cell source for TE is of critical importance for success. For skeletal applications, cells may be required to survive in an artificial construct introduced into an inflamed environment. At the same time, they must be able to respond to positive cues determining that they remain as, or develop into, the required cell type. Cells need to be able to respond to biomechanical cues to produce the structure and function of the original tissue or organ, which means they must interact with other cells and migrate appropriately, or avoid migrating, as their destiny dictates. Cells that are plastic and amenable to such cues are likely to be committed progenitors. So where do we obtain such cells? Adult mesenchymal stromal cells (MSCs) have been seen as an attractive source of cells for skeletal tissues. They can be extracted from the bone marrow or peripheral blood, and have multi-lineage differentiation ability into osteogenic, chondrogenic or adipogenic cell types. However, these cells have disadvantages in their reduced differentiation potential on expansion and lack of long-term retention in vivo [5–7] combined with the need for several operations for autologous transplantation and challenges in using them allogeneically, which may hinder their...
widespread clinical application. Notably, they also play immunomodulatory roles. An alternative and attractive cell source for TE is human pluripotent stem cells (hPSCs). These are either human embryonic stem cells (hESCs), derived from the inner cell mass of the human blastocyst surplus to assisted reproduction programs, or human induced pluripotent stem cells (hiPSCs), which are reprogrammed somatic cells. They combine a unique ability to generate virtually any cell type in the body (i.e., pluripotency) with continuous replication without differentiation (i.e., self-renewal) and are becoming increasingly relevant to modern medicine. Importantly, there are now thousands of lines worldwide including some derived and banked at clinical grade [8,9] and therefore usable for cell therapies. With increasingly robust differentiation protocols available, hPSCs hold great promise as biological starting materials for a multitude of applications including regenerative medicine, disease modeling and drug development. Herein we review the available protocols for hPSC skeletogenesis with a focus on cartilage and bone. We discuss some of the most recent advances in enabling technologies and how their integration with hPSC differentiation will eventually lead to a new generation of bioengineered tissues for skeletal regeneration.

**Routes for hPSC differentiation into skeletal tissues**

Methods to generate skeletal tissues from hPSCs primarily follow one of three routes: (1) Initially nonspecific differentiation, in which the minimal signals required to drive skeletogenesis are applied in a simple and cost-efficient manner; (2) Generation of MSC-like cells, serving as an alternative to adult MSCs; (3) Developmentally-guided differentiation, which recapitulates multiple steps of embryonic development in vitro with the aim to generate authentic skeletal cell types of specific regions of the body. These methods are discussed below, and their advantages and limitations are summarized in Table 1.

### Nonspecific initial differentiation

Nonspecific initial differentiation protocols for hPSC skeletogenesis follow one of four strategies: (A) Monolayer differentiation; (B) Formation of embryoid bodies; (C) hPSC micromass cultures, in each case followed by chondro- or osteo-genic differentiation or; (D) hPSC differentiation through co-culture with adult target cell types (Figure 1).

Nonspecific differentiation as a monolayer (Figure 1(A1)) is a simple strategy that involves the treatment of hPSC monolayers with a differentiation medium in order to generate a population of differentiated cells in a single step, without sequentially driving multiple stages of development. Promising hPSC differentiation protocols using this method are scarce, but in recent years protocols for osteogenesis have been reported [10,11]. One method involves the direct application of osteogenic medium [Minimum Essential Medium Eagle – alpha modification, fetal bovine serum (FBS), nonessential amino acids, \( \beta \)-mercaptoethanol, ascorbic acid, sodium glycerophosphate and dexamethasone (DEX)] for 35 days. Despite enhanced expression of osteogenic markers, this strategy resulted in a highly heterogeneous population of cells, and low differentiation efficiency, evidencing the need for more specific methods [10]. A different strategy uses osteogenic medium supplemented with retinoic acid (RA), resulting in the formation of calcified osteogenic nodules, from which osteogenic cells can be isolated. The use of RA was shown to stimulate BMP and WNT signaling, allowing cells to differentiate further when compared to osteogenic medium alone, and triggering the expression of osteocyte markers such as PHEX and sclerostin [11].

The use of embryoid bodies is a common strategy to promote hPSC differentiation into cells equivalent to the ectoderm, mesoderm, and endoderm – the three
Figure 1. Strategies for nonspecific differentiation of hPSCs. (A) Nonspecific differentiation as a monolayer through treatment of PSCs with differentiation medium is an uncommon strategy but has been applied to osteogenic differentiation [10,11]. (B) Embryoid body (EB) formation enables spontaneous differentiation of the three embryonic germ layers. Continuous culture of EBs within chondrogenic differentiation medium supplemented with GFs may lead to generation of cartilaginous cell types [12,13]. Alternatively, EB culture with osteogenic medium leads to generation of mineralizing osteoblastic-like cells [14]. (C) Micromass culture involves condensation of PSCs within a small volume of chondrogenic growth medium in the presence of GFs. Chondrogenic-like cells then can generate cartilaginous tissue within scaffold-free pellet culture [15]. (D) Co-culture systems require the presence of adult primary cells to influence PSC differentiation through paracrine cues. PSCs can be separated from primary cells via a porous membrane that enables the passage of chemical cues (top) or mixed within micromass cultures micromass cultures (bottom), which requires a selection method to remove primary cells post-differentiation [16–18]. Days noted represent period of time spent in appropriate culture as illustrated.
embryonic germ layers. After embryo body formation, different levels of control over differentiation of the mesoderm toward skeletal lineages can be exerted. The simplest methods involve treatment with chondrogenic, or osteogenic medium, which can be supplemented with growth factors (GFs), particularly those of the TGFβ superfamily for chondrogenesis, or ascorbic acid, β-glycerophosphate, and DEX for osteogenesis (Figure 1(B)) [12,13]. Multi-stage differentiation protocols using embryoid bodies, but aiming to recapitulate different stages of development, or including a mesenchymal stem cell-like stage, have also been established (Sections “hPSC-derived MSC-like Cells” and “Developmentally-guided Differentiation”).

Similarly to embryoid bodies, differentiation can be promoted in hPSC micromass cultures by treatment with chondrogenic GFs such as BMP-4, which was shown to result in the generation of cells expressing collagen type-II, a key cartilage ECM component (Figure 1(C)) [15]. However, the efficiency of these systems is often low, with one study reporting approximately 10% of cells in the micromass expressing COL2A1 promoter-driven GFP. Thus, cell sorting was required to obtain appropriate cells for further chondrogenic differentiation, first as a monolayer treated with FBS, and then as pellet cultures exposed to TFGβ-3 [15].

A third method of differentiation is the use of co-culture systems, in which adult primary cells, or cell lines, promote differentiation of hPSCs into the cell type of interest. These systems have been developed using chondrocytes [16,18], or limb bud progenitor cells from mouse embryos [17]. In order to ensure that the resulting cell population is homogeneous and consists of hPSC-derived progeny, the cells used as a co-culture can be separated in the culture well by a porous membrane (Figure 1(D top)) [16], or cell division of the co-cultured cells can be inactivated through gamma irradiation [18,19]. Alternatively, hPSCs can be transfected with genes for antibiotic resistance and selected after exposure to the antibiotic to purify the final hPSC-generated population (Figure 1(D bottom)) [17].

Despite their ease of use, nonspecific differentiation methods often have low efficiencies, and the mechanisms guiding differentiation are less defined. Co-culture systems rely on the availability and homogeneity of differentiated cell types, which likely promote differentiation through the release of paracrine factors or cell-cell signaling, but may be affected by batch-to-batch or donor variability. However, these systems can still be valuable due to their simplicity and used to further interrogate the signals needed for hPSC differentiation. The identification of the paracrine factors released by cells in co-culture and their mode of action may lead to the generation of new methods to differentiate hPSCs into skeletal lineages, by replacing the co-culture with a medium containing key GFs identified, or small molecules.

**hPSC-derived MSC-like cells**

The use of MSCs is a widely adopted strategy for skeletal TE. hPSCs can serve as a cell source for the generation of MSC-like cells, often called induced mesenchymal stem cells (iMSCs) [20]. Despite their origin, iMSCs meet some of the minimal criteria for MSC classification as defined by the International Society for Cellular Therapy [21] although adipogenic differentiation is not a requirement for cells to give skeletal lineages.

The generation of iMSCs is one of the simplest and most widely used methods for the generation of cartilage and bone from hPSCs (Figure 2). Through a sequential multistage process, hPSCs are initially differentiated either through embryoid bodies [22,23], or monolayer culture. Further culture with medium containing FBS leads to the formation of MSC-like cells. Finally, chondro- or osteogenic differentiation is induced in a similar manner to that for adult MSCs. Chondrogenic differentiation can be achieved through the use of commercially available chondrocyte differentiation medium [24], or through supplementation with chondrogenic GFs such as TGFβ-3 [25], TGFβ-1 [22], or BMP-2 [26]. Osteogenesis can be promoted by the use of commercially available osteogenic medium [27], or media supplemented with dexamethasone, β-glycerophosphate, and ascorbic acid-2-phosphate [22,28,29]. Indeed, although less investigated, tenocytes for tendon repair are also obtainable through stimulation with FGF2 and TGF-β [20,30].

When compared to adult MSCs, the generation of iMSCs requires an additional mesenchymal differentiation step. However, their use can offer multiple advantages. Unlike adult MSCs, hPSCs can self-renew *in vitro* for multiple passages, overcoming supply limitations [31,32]. In addition, hiPSC generation from peripheral blood [33] or skin cells [34] can enable the generation of high iMSC cell numbers, with reduced donor site morbidity and variability [35]. When considering the use of iMSCs for chondrogenesis, it is important to note that the resulting cell type is likely representative of a prehypertrophic growth plate chondrocyte, characterized by expression of markers such as SOX9, IHH, RUNX2/3, collagen type II, and type X [26,36]. Growth plate chondrocytes are known to undergo hypertrophy.
and participate in the process of bone formation by endochondral ossification, being replaced by osteoblasts but also contributing directly to osteogenesis [37–39]. However, this means that iMSCs’ use for articular cartilage (AC) applications may be more limited.

**Developmentally-guided differentiation**

In vivo, skeletal tissues can have neuroectodermal or mesodermal origin, depending on their anatomic location (Figure 3(A)). It is important to understand how these tissues develop, and to be aware of the signals needed for cell lineage commitment, in order to inform selection of the best differentiation protocol. The craniofacial skeleton is mainly an ectodermal neural crest derivative, but most bone and cartilaginous tissues in the body originate from the mesoderm. The remaining axial skeleton originates predominantly from the paraxial mesoderm through the somites, whereas the lateral plate mesoderm (LPM) gives rise to the cartilage, tendons, and bone of the appendicular skeleton [49]. Within the fetal limb, different types of cartilage develop: growth plate cartilage, which serves as a template for bone development, and AC, which lines the ends of long bones at the joints.

Most developmentally-guided hPSC differentiation methods use combinations of GFs and small molecules to stimulate or inhibit cell-signaling pathways that play a crucial role in development. The most widely used stimulatory/inhibitory factors are summarized in Table 2 and can be used by tissue engineers as a toolbox to guide hPSC differentiation into skeletal lineages. By using these factors, developmentally-guided hPSC differentiation protocols have been developed, following the three main routes: neural crest, paraxial mesoderm, and lateral plate mesoderm. The most important signals
needed to promote hPSC differentiation toward these lineages are outlined in Figure 3(B), and can serve as a guide toward the development of improved methods of differentiation.

**Ectoderm: Neural crest**

Gastrulation specifies pluripotent cells from the inner cell mass into the three germ layers: the ectoderm, mesoderm, and endoderm. The first step in gastrulation

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**Figure 3.** Overview and roadmap of skeletal tissue derivatives. (A) Different embryonic regions give rise to distinct parts of the adult skeleton: the neural crest generates head and neck cartilage and bone; the paraxial mesoderm gives rise to the axial skeleton; the lateral plate mesoderm originates the appendicular skeleton. (B) Roadmap of mammalian skeletal development with reference to key papers that illustrate derivation or identification of specific skeletal lineages from PSCs. Activation or inhibition of cell signaling pathways required to drive differentiation of each lineage outlined as described in key references [40–48].
is the formation of the primitive streak, a transient structure through which mesoderm and endodermal precursors are internalized under the prospective ectoderm [66,67]. At this stage, pluripotent cells commit to differentiation toward ectoderm or mesendodermal fates.

The neur ectoderm gives rise to the neural crest, which generates skeletal components of the head and neck (Figure 3(A)) [68]. HPSCs can be differentiated into skeletal tissue types following the neural crest and ectomesenchymal cell stages. One method to generate neural crest precursor cells is through their isolation from neural rosettes followed by FACS sorting (precursors HNK1^+ p75^+ ) [69]. A different strategy uses WNT stimulation through GSK3 beta inhibition, and inhibition of BMP and Activin/Nodal [70–73]. Neural crest cells can then be differentiated into mesenchymal cells through culture in an FBS-rich medium or in a neural crest induction medium. Finally, mesenchymal cells can be further differentiated using chondrogenic medium supplemented with BMP-4 (and in later work BMP-2), while follistatin was employed to inhibit endoderm formation. More recently, a simpler method was developed, using only CHIR99021, a WNT activator, and TTNPB, a retinoic acid receptor pan-agonist. The resulting cells expressed key chondrogenic markers, both in vitro and in vivo after subcutaneous implantation in mice [62]. The exact mechanism of action of TTNPB remains unclear, but it may act as an epigenetic modulator.

As different regions of the mesoderm can generate skeletal tissues of distinct regions of the body, acquisition of a particular mesoderm phenotype is important. The specification of a paraxial vs. lateral plate phenotype is heavily reliant on a balance between WNT and BMP signaling. WNT stimulation promotes paraxial mesoderm whereas BMP leads to lateral plate mesoderm. Conversely, inhibition of either of these signals can induce the generation of the opposite mesodermal fate [43,73].

**Mesoderm**

During gastrulation, mesendodermal precursors formed in the primitive streak are able to generate both mesodermal and endodermal progenitors [74]. This process, replicated in vitro, forms the basis for differentiation protocols that follow a two or three-step strategy, differentiating hPSCs first through a primitive streak-like stage via epithelial-mesenchymal transition (EMT), with formation of mesendoderm and mesoderm and then toward a chondrogenic phenotype [55,59,62]. In vivo studies of embryonic development have uncovered the crucial importance of TGFβ, WNT, FGF, and BMP signaling in primitive streak induction [75–78]. Strategies focused on in vitro differentiation of hPSCs into primitive streak-like cells often employ GFs or small molecules that stimulate these pathways.

Pioneering work has promoted mesendoderm induction using combinations of WNT, Activin, and BMP stimulation [79]. Then, a mesoderm phenotype was achieved through the use of BMP-4 and in later work BMP-2, while follistatin was employed to inhibit endoderm formation. Finally, chondrogenesis was promoted using a switch from BMP-4/2 to GDF-5 [55,59]. More recently, a simpler method was developed, using only CHIR99021, a WNT activator, and TTNPB, a retinoic acid receptor pan-agonist. The resulting cells expressed key chondrogenic markers, both in vitro and in vivo after subcutaneous implantation in mice [62]. The exact mechanism of action of TTNPB remains unclear, but it may act as an epigenetic modulator.

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**Paraxial mesoderm**

After paraxial mesoderm induction, hPSC-derived cells can be directly differentiated into osteoprogenitor cells
through treatment with osteogenic medium [73]. Alternatively, paraxial mesoderm-like cells can be differentiated into an early somite phenotype through WNT stimulation combined with inhibition of FGF, TGFβ, and BMP signaling. Then, WNT and hedgehog signaling drive the bifurcation between ventral (sclerotome) and dorsal (dermomyotome) portions of the somites. By stimulating WNT and inhibiting hedgehog signaling, sclerotome-like cells can be produced, which differentiate into skeletal fates including osteo- and chondroprogenitors. Sclerotome progenitor implantation in vivo leads to ectopic bone formation whereas stimulation with BMP can generate a pool of highly chondrogenic cells [43]. Additional treatment with WNT inhibitor CS9 has been shown to further enhance chondrogenesis using this system [80]. However, despite the development of highly efficient, multi-step differentiation protocols, generation of homogeneous cell populations is still challenging. Single-cell transcriptomic analysis has revealed that this protocol still generates some off-target cell types such as neural cells and melanocytes during such paraxial chondrogenic differentiation [80].

**Lateral plate mesoderm (LPM)**

Efforts to map LPM development using hPSCs were pioneered by Loh and colleagues, with the identification of WNT stimulation as key to induce a limb bud-like phenotype, with increased expression of PRRX1 and HOXB5, whereas WNT inhibition resulted in cardiac differentiation [43]. When WNT stimulation is combined with sonic hedgehog activation, PSC-derived LPM cells can be differentiated into tracheal cartilage and smooth muscle cells [42]. However, when RA is applied under WNT inhibition, different splanchic lateral mesoderm progeny can be formed while inhibiting the formation of anterior LPM-derived cardiomyocytes [41]. In vitro, RA signaling modulation has been used to specify forelimb vs. hindlimb phenotype using mouse PSC aggregates in suspension culture. Exposure to RA resulted in upregulation of forelimb marker Tbx5, whereas the use of a RA antagonist resulted in increased expression of hindlimb-specific Tbx4 and Pitx1 [48]. Despite significant progress in the field, the full range of necessary signals for the generation of skeletal tissues from LPM and limb-bud intermediates using hPSCs is still unclear and single-cell analysis has not been published. However, recent work has shown the generation of osteoprogenitor cells from an LPM-like intermediate through treatment with osteogenic medium [73], and chondro- and osteogenic cells from a limb bud-like intermediate through the activation of BMP and TGFβ, or WNT signaling respectively [47].

**Enabling technologies**

The stem cell engineering field is rapidly evolving, thanks to a clearer understanding of the temporal dynamics of stimulation and inhibition of signaling pathways that enable the generation of desired cell types. However, current methods for hPSC skeletal differentiation are still heavily reliant on the use of GFs, which are costly, suffer from batch to batch variation and have short shelf lives and half-lives in vitro. Protocols are frequently conducted in 2D, or in simple 3D models such as embryoid bodies or cell spheroids of limited size. In contrast, technological innovations have emerged in closely related fields such as biomedical engineering, nanotechnology, and synthetic biology. Even though some of these have found applications in the field of TE, their potential has not yet been fully explored in the generation of skeletal tissues from hPSCs. The opportunities they offer for improving methods of generation of cartilage and bone from hPSCs are summarized in Figure 4.

**Cell selection**

Spontaneous differentiation of hPSCs and poor efficiency of differentiation protocols can lead to unpredictable and heterogeneous off-target populations of non-chondrogenic or osteogenic cells. Skeletal progenitors are cells capable of selectively giving rise to bone, cartilage, and stromal cells. Efforts to identify them have been made in recent years [81–84]. However, a gold standard combination of markers has not been definitively agreed upon. Researchers have mainly investigated such markers in rodents [82,83,85] but occasionally human fetal limb [86] or adult joints [87] have been the source. A list of potential markers for the identification of overall skeletal progenitors as well as osteogenic and chondrogenic progenitors is reported in Table 3. The need for combinations of markers is very clear.

One goal has been to use selection methods to isolate such progenitors for further differentiation thus improving the purity level of mature skeletal populations. The selection process has to retain cell viability and progenitor characteristics in order to continue to allow healthy culture and further differentiation after sorting. The latter has to avoid damage to the cells precluding the use of intracellular markers which would require fixation and permeabilisation. However, intracellular markers are still exploitable if gene-reporter lines can be engineered without interfering with cell phenotype. Currently, Fluorescent-Activated Cell Sorting (FACS), the gentler Magnetic-Activated Cell Sorting
(MACS) and Microfluidic cell sorting technologies (MST) are the main techniques adopted for cell selection (Table 4). In FACS, cells are bound to fluorescent antibodies to particular lineage-selective markers (or engineered with fluorescent lineage-selective promoter-reporters) and sorted according to label intensity with gating selected by the operator [92]. MACS still requires the use of antibodies, but uses magnetic particle-bound antibodies. Cells positive for the selected antigen bind to the antibody and through the use of a strong magnet are retained, whereas negative cells can be washed away. Alternatively to FACS and MACS, in MST, antibodies are not required, and the label-free cells are sorted based solely on size and morphology. This was
Illustrated in a recent publication, where undifferentiated MSCs were sorted through the use of a spiral microfluidic chip into three different subpopulations according to size [93]. Employing gene expression and staining techniques, the authors were able to identify medium and large size cells as being more chondrogenic (pre and post differentiation) and osteogenic, respectively. Single-cell RNAseq analysis may confirm and extend such marker selections [80,91].

**Biofabrication strategies**

Biofabrication is the automated generation of living functional products [94] and comprises *top-down* or *bottom-up* strategies. *Top-down* strategies are characterized by the fabrication of temporary structures (i.e., scaffolds) to support and guide tissue formation by seeded cells. *Bottom-up* approaches require the use of cells as building blocks (i.e., spheroids, sheets, cell-laden hydrogels) to generate tissue constructs with high degree of complexity through automated assembly processes. *Top-down* strategies such as the use of nanofibrous scaffolds have been shown to improve osteogenic differentiation from iPSC-derived embryoid bodies [95,96] and iMSCs [97]. Similarly, culture of iMSCs within decellularized bone scaffolds has resulted in improved osteogenesis *in vitro* and *in vivo*, with enhanced tissue maturation after implantation [98]. *Bottom-up* approaches such as 3D bioprinting have also been used for hPSC skeletogenesis. Nanofibrillated

| Markers | Differentiating fate | Cell source | Additional information | Reference |
|---------|----------------------|-------------|------------------------|-----------|
| CD105⁺ CD73⁺ CD90⁻ | Osteogenic, Chondrogenic, | Human embryonic | Minimal criteria for defining MSCs | [21] |
| CD45⁻ | Adipogenic | | | |
| CD90⁺ HLA-DR⁺ | | | | |
| CD166low/mid, CD146⁺ | Chondrogenic | Human | Potential human chondrocyte | [86] |
| CD73⁺ (CD29⁺ CD31⁻) | | long bones | progenitors | |
| CD34⁻ CD45⁻ | | | After exclusion of endothelial, epithelial and | |
| CD235⁻ CD44low | | | hematopoietic cells | |
| CD166low/mid BMPR1B⁺ | Chondrogenic | hESCs | Potential prechondrogenic cells | |
| CD45⁻ TER119⁺ CD202b⁺ | Multipotent | Mouse Limb | Mouse skeletal stem cells. | |
| AlphaV⁺ CD90⁻ 6C3⁻ | | | Potential to form cartilage, bone and stroma. | |
| CD45⁻ TER119⁺ CD202b⁺ | Multipotent | Mouse Limb | Mouse osteoprogenitors, chondroprogenitors, stromal | |
| AlphaV⁺ CD90⁻ 6C3⁻ CD105⁻ | | | progenitors | |
| CD45⁻ TER119⁺ CD202b⁺ | Multipotent | Mouse Limb | Human chondroprogenitors, no | |
| AlphaV⁺ CD90⁻ 6C3⁻ CD105⁻ CD200⁺ | | | bone or stroma formed after | |
| | | | *in vivo* implantation | |
| CD105⁺ CD146⁺ | Osteogenic | Rat | Rats Osteoprogenitors | [85] |
| CD146⁺ | Chondrogenic | Rat | Rats Chondroprogenitors | [85] |
| PDPN⁺ CD146⁺ | Osteogenic | Human fetal growth plate | Human osteoprogenitors, no | |
| CD90⁻ | | | cartilage or stroma formed | |
| after *in vivo* implantation | | | | |
| PDPN⁺ CD146⁺ CD73⁻ CD164⁺ | Chondrogenic | Human growth plate | Human chondroprogenitors, no | |
| | | | bone or stroma formed after | |
| | | | *in vivo* implantation | |
| PDPN⁺ CD146⁺ CD73⁻ CD164⁺ | Multipotent | iPSCs | Human skeletal stem cells. | |
| | | | Differentiated in cartilage or bone in vitro, formed ossicles when | |
| | | | implanted in vivo. No stroma formation. | |
| PDPN⁺ CD146⁺ CD73⁻ CD164⁺ | Chondrogenic | iPSCs | Human Chondroprogenitors | [87] |
| CD164⁺ | | | | |
| ITG4⁺ BMPR1B⁺ | Osteogenic | Adult pig knee | High expression of RUNX2 | |
| ITG4⁺ BMPR1B⁺ | Chondrogenic | Adult Pig knee | High zone and terminal | |
| | | | differentiation status | |
| ITG4⁺ BMPR1B⁺ | Chondrogenic | Adult Pig Knee | Transitional zone chondrocytes | |
| CD49f⁺ | Multipotent | hESCs and hiPSCs | Suggested marker for skeletal | |
| PDGFRb⁺ CD146⁺ CD166⁻ CD45⁻ | Chondrogenic | hiPSCs | Cell sorted using a COL2A1-GFP | |
| PDGFRα⁺/⁻ PDPN⁺ CADM1⁺ | Osteogenic Chondrogenic | Embryonic and fetal human | No adipogenic differentiation. No | |
| PDGFRα⁺/⁻ PDPN⁺ CADM1⁻ | Osteogenic | Embryonic and fetal human | hematopoietic potential in *vivo*. | |
| | | long bones | | |
| | | | | |

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cellulose (NFC)-based hydrogels were developed as bioinks to promote the chondrogenic differentiation of encapsulated iPSCs. Comparison between different bioinks suggested increased levels of cell proliferation when NFC was blended with alginate compared to blending with hyaluronan, with cells retaining the expression of chondrogenic markers in 3D culture [18].

Despite these examples, the use of biomaterials and biofabrication technologies for skeletal TE applications using hPSCs has been limited thus far, and their full potential is yet to be explored. In order to achieve control over PSC differentiation in biofabricated constructs, stringent design and manufacturing requirements need to be considered and can be found elsewhere [99]. However, 3D bioprinting can offer multiple advantages, such as: (1) Control over macroarchitecture, allowing the fabrication of patient-specific constructs. (2) Control of microarchitecture, regarding pore size, shape and interconnectivity, and ability to harness the extrusion process to orient microfibers within a bioink [100]. (3) Integration of multiple materials within a single construct, providing the ability to mechanically reinforce weak hydrogels with stiffer materials such as thermoplastics [101]. (4) Ability to integrate multiple cell types and generate heterogeneous tissues, such as the osteochondral interface [102]. Due to these advantages, the integration of biofabrication technologies with hPSC differentiation methods can offer innovative ways to control cell differentiation, reproduce cell niches, and maintain cells with a stable phenotype to generate three-dimensional skeletal tissue constructs.

**Advanced bioreactors**

During development, both biomolecular cues and biomechanical forces are instrumental in influencing skeletal cell fate and differentiation. Cell response to load through integrins, mechanoreceptors, and primary cilia are critical for endochondral skeleton development [103,104] and articular cartilage homeostasis [105,106]. In 3D cartilage constructs and stem cell protocols, moderate cyclic hydrostatic pressure (around 3–10 MPa and 1 Hz) has been shown to increase collagen II and GAG deposition and the chondrogenic phenotype in newly forming cartilage [107–109]. While bone is mainly exposed to cyclic compression, articular cartilage also has to withstand shear stresses. The use of advanced bioreactors offers the ability to replicate these conditions in vitro through the application of multiple physical stimuli including compression, shear and hydrostatic pressure (HP). These stimuli can be applied through the solid (e.g., scaffold) or liquid phases (e.g., cell culture medium), either separately or in combination. Compression and shear stresses are generally deployed on the solid construct by direct mechanical loading, whilst HP and fluid shear can be applied to cells by tuning the flow parameters of the liquid medium (i.e., velocity, pressure, etc.). Application of mechanical stimuli on hPSC skeletal differentiation has shown promising results in guiding cell fate. For cartilage TE applications, the use of cyclic compression can limit hypertrophic differentiation of iMSCs embedded in 3D hydrogels, reducing collagen type-X [54]. A distinct application of bioreactors is its miniaturization with the integration of microfluidics and organ-on-a-chip technologies. Microfluidic bioreactors enable cell culture with smaller medium volumes, reducing the high cost of supplements for hPSC differentiation. High-throughput systems can make it easier to evaluate the effects of different concentrations or combinations of biochemical compounds [110]. In addition, the ability to perform mechanical stimulation within microfluidic systems has made it possible to study the response of chondrocytes at the onset of osteoarthritis to compression [111]. Harnessing the full potential of bioreactor technologies by integrating them with hPSC differentiation systems may lead to new ways of guiding cell differentiation toward specific developmental lineages, and to a greater understanding of mechanotransduction processes.

**Characterization methods**

Classically, in vitro characterization of skeletogenesis in scaffold-based or scaffold-free systems has involved the
use of histochemical stains such as Safranin O or Alcian/Toluidine blue for sulfated GAGs, Picrosirius red for collagen fibers and Alizarin red for mineralized tissue [112], together with immunocytochemistry of fixed tissue for key proteins. Picrosirius red staining is best viewed under polarizing light microscopy where the birefringence reflects collagen bundle alignment.

These are still the cornerstone of the characterization repertoire but recently many more techniques have emerged. It is now standard to assess gene expression using quantitative reverse transcription PCR (RT-qPCR) for transcription factor and matrix molecule genes expressed during chondrocyte development [43,55,113] or in mature chondrocytes or osteoblasts [114,115]. This can be extended to assessment of the whole transcriptome through RNAseq [116], e.g., comparison with developing limb tissue [88], osteogenesis compared to calvaria [115] and evaluation of chondrogenesis-associated microRNAs [116,117]. More recently it has been possible to delve into the nature of the different cells developing to form scaffold-free cartilage using single-cell RNAseq [80]. This allows the identification of off-target cells that would be detrimental to the generation of homogeneous engineered tissue. It also allows detailed cell evaluation in comparison to native tissues in which a number of cell types are present, such as in bone.

Mass spectrometry (MS)-based methods allow for evaluation of bimolecular sample composition, making it possible to conduct proteomic or lipidomic studies. The use of proteomics has been applied to analyze the composition of cartilage [118] and bone [119], as well as their changes with disease such as in osteoarthritis [120,121]. The main advantage of these techniques over most methods is the untargeted analysis, and the ability to identify post-translational modifications such as phosphorylation, important in the regulation of protein function [122]. MS-based proteomics has been applied to study bone and cartilage, both at the tissue and cellular levels. Using this technology it is possible to identify both intracellular and extracellular proteins such as collagens, proteoglycans, osteocalcin and BMPs [123]. The first proteomic map of human articular chondrocytes in culture resulted in the identification of key proteins with roles in cellular organization, metabolic activity, ECM production and remodeling [118]. Studies with hESCs have revealed important insights about how they change during differentiation [124]. LC-MS/MS has also been applied to characterize the secretome of hESC-derived iMSCs, allowing the identification of 247 proteins differentially present in conditioned medium, which might provide valuable insight about their paracrine effects [125]. Recent developments in mass spectrometry have enabled application in the analysis of tissue sections with spatial resolution. This technology made it possible to analyze the protein or lipid distribution without the need for targeted approaches such as the use of antibodies [126,127]. Despite limited applications in skeletal TE [127], mass spectrometry imaging may become a powerful technology for sample analysis, with particular benefits when considering interfacial tissues such as the osteochondral interface.

Mechanical characterization tools provide vital information on the physical properties of TE constructs regulating skeletogenesis, and determine their suitability to support tissue growth under physiological loads. Therefore, characterizing the mechanical properties of skeletal TE implants is an important parameter in their development. Mechanical characterization typically involves the use of static mechanical force testers to measure the compressive or tensile properties of the scaffold (e.g., Young’s modulus, tensile strength, compressive modulus or compressive strength) [96,128–131]. Compressive modulus is frequently used as an indirect method of measuring the scaffold degradation rate, by measuring repeatedly at time intervals [128,129,132]. While cartilage applications typically focus on compression testing, tensile testing is an important parameter for bone constructs in order to prevent bone fracture. Rheological measurements (i.e., viscosity, recovery, storage, and loss modulus, etc.) are also very common, especially for cartilage applications where polymeric hydrogels are increasingly used to mimic the viscoelastic nature of the native tissue [133,134].

**In silico models**

Despite the importance of *in vitro* and *in vivo* studies to assess the performance of hPSC-derived cells for skeletogenesis, the use of computational *in silico* models has been gaining relevance as a tool to predict cell behavior and quantitatively simulate experimental scenarios that might lead to an accelerated route to market with increased speed and reduced costs.

In a two-part study, Campbell and coworkers propose a reaction-diffusion mathematical model to investigate the effect of GFs (BMP-2 and FGF-1) and co-implantation (i.e., autologous chondrocytes and MSCs) on cartilage regeneration after cell therapy [135,136]. The simulation results showed differences in matrix production, distinct effects of the different GFs, and suggested an optimal cell ratio for co-implantation of...
autologous chondrocytes and MSCs in order to accelerate cartilage repair and lead to higher matrix densities.

Employing a mathematical model based on ordinary differential equations, Gaspari et al. investigated the complex paracrine mechanisms associated with mesendodermal differentiation of hPSCs. The authors suggested that at least three paracrine factors, LEFTY1, CER1, and an undefined activator must be included in the simulation to accurately replicate the in vitro differentiation kinetics of hPSCs [137].

A different model [138] recapitulated in vitro stem cell morphogenesis in silico and demonstrated the possibility of controlling multicellular patterning toward the generation of human organoids and tissues. In an attempt to overcome some of the limitations associated with the in vitro culture of hPSCs and to scale-up their bioprocessing, Manstein and colleagues have combined instrumented stirred tank bioreactor technology with in silico modeling [139]. The authors demonstrate that a 70-fold cell expansion in 7 days (independently of the cell line) is achievable while pluripotency, differentiation potential and karyotype remain unaffected. The proposed strategy also allows for significant economic savings (over 75% reduction in medium consumption) which, in combination with the large cell volumes yielded, represent an important step toward the mass production of hPSCs for clinical implantation. Taken together, the above studies demonstrate how in silico modeling can be used to inform hPSC technology and eventually drive the future of regenerative medicine therapies. However, this approach has yet to be applied to differentiation of hPSCs toward skeletal lineages or the application of chondro- or osteoprogenitors in tissue repair.

**Stimulation of growth factor signalling**

Growth factors are critical components of hPSC-skeletal engineering strategies due to their key role in directing cell fate. However, the clinical application of GFs is limited by their short half-life and rapid denaturation in vivo, which leads to poor local retention and consequent supraphysiological dose requirements [140]. The dosage-related adverse effects of GFs used for bone or cartilage repair have included osteoclast activation [141], ectopic bone formation [142], adipogenesis [142], fibrosis and hypertrophic scars [143]. When using scaffolds for tissue repair, the controlled and sustained delivery of GFs to cells within any 3D construct is crucial. This has driven the development of controlled-release biomaterial-delivery systems designed to retain active GFs for longer and allow specific GF targeting while avoiding the adverse effects of inappropriate concentrations.

Skeletogenic delivery systems have incorporated different GFs namely BMP2 [144], BMP7 [145], TGFβ-3 [128,134,145], VEGF, and PDGF [146] to promote cartilage or bone regeneration and the vascularization of the latter. GF release profiles and encapsulation efficiency are most commonly measured using an Enzyme-Linked Immunosorbent Assay (ELISA) [128,134,144,145,147]. Direct physical encapsulation of the GF within the scaffold matrix is the simplest delivery strategy. In such systems release kinetics are determined by the biodegradation of hydrophobic polymers (e.g., poly-lactide-co-glycolide (PLGA) [148], polycaprolactone (PCL) [149]), or diffusion of GF through porous hydrogel networks such as chitosan [150], and alginate [151]. The latter is used frequently, but limited by the high porosity and hydrophilicity of the hydrogel matrix, resulting in poor retention of the GF and early burst release which leads to, e.g., chondrocyte hypertrophy [152].

Triggered delivery mechanisms via electro-responsive BMP-2 release and TGFβ immobilized magnetic beads have been developed for greater spatiotemporal control over the release profile [144,153]. Nanoparticle tethering and microsphere encapsulation are increasingly used to obviate burst release kinetics by acting as secondary carriers [128,134,145–148,154]. Indeed, Zhou et al. reported <0.35% release of TGFβ-3 from graphene oxide nanoflakes after 72 h [134]. Dual delivery systems have also been developed to better mimic the stimulation of parallel complex signaling pathways which drive the development of skeletal tissues in vivo [145,146]. Currently, the spatiotemporal delivery of GFs to encapsulated cells within 3D constructs has yet to be explored with pluripotent cell types.

**Optogenetic technologies**

Although tissue morphogenesis is a dynamic process, in vitro recapitulation of developmental processes during hPSC differentiation is limited by the intrinsic properties of stimulation by GFs (Section “Stimulation of growth factor signalling”). Addition of exogenous factors, including GFs and small molecule agonists, cannot accurately reflect the dynamic signaling cues that cells are subject to in vivo – both in time and space. The emergence of optogenetic approaches provides a means to develop synthetic photoreceptors that enable dynamic manipulation of cell signaling pathways with spatiotemporal precision [155]. Optogenetics describes combining light-sensitive molecules with cell signaling machinery that then renders cell signaling activity under control of a
specific light wavelength (Figure 4). Using this approach light can be used to drive the signaling pathways normally triggered selectively by, e.g., specific chondro- or osteogenic growth factors. Further details on optogenetic technologies and their developmental implementation can be found elsewhere [156–160].

Incorporation of optogenetic tools within TE is currently in its infancy, but there is great potential for combining technologies to enhance current approaches (reviewed [161,162]). Signaling pathways crucial for skeletal development and differentiation of hPSCs/MSCs include the TGFβ and BMP signaling pathways (Sections “Developmentally-guided Differentiation” and “Stimulation of Growth factor Signalling”). Targeted optogenetic manipulation of both pathways to investigate early developmental processes in vivo [163,164], which control intracellular signaling dynamics [165] and elicit downstream transcriptional activity in hPSCs [166], has been described in recent years. Additionally, optogenetic induction of BMP-2 gene expression within MSCs has been demonstrated to enable control of osteogenic fate in vitro and fine-tune bone regeneration in vivo [167]. Such reports illustrate the potential for integrating optogenetic technologies within skeletal differentiation approaches. Their future use will improve hPSC differentiation consistency and enable control of regionalized cell signaling cues within 3D-stratified constructs.

Conclusions and outlook

The advent of stem cell technologies has led to major breakthroughs in modern medicine, with promising results in the regeneration of multiple tissue types, including the skeletal system. Multiple stem cell-based therapies are now reaching clinical trials [168], including adult MSCs for the treatment of osteoarthritis and bone lesions [169].

The establishment of methods to efficiently differentiate hPSCs into skeletal lineages such as chondrogenic, osteogenic or tenogenic-like cells has led to their identification as a promising cell source for orthopedic applications due to their availability, expansion potential, and potency when compared to adult MSCs or alternative cell types. From a technical perspective, and although many differentiation protocols are available, the reproducibility, specificity, scalability and associated costs of using hPSCs still limit their clinical translation. In order to generate cell types that closely resemble native tissues, hPSC differentiation protocols can be designed to follow the developmental routes that generate the authentic tissue types in the embryo and fetus. This strategy is likely to result in improved differentiation stability and more homogeneous cell populations. However, developmentally-guided differentiation protocols are still heavily reliant on costly reagents such as GFs. The use of improved delivery methods and the identification of suitable alternatives such as small molecule analogues, or the use of innovative technologies such as cell sorting or optogenetics, is expected to lead to cost reductions and improved efficiencies for TE.

In order to repair relevant clinical defects, large-scale 3D tissues are needed. However, most hPSC differentiation protocols have been developed as 2D monolayer cultures and lack validation in 3D. The integration of biofabrication technologies to reproduce the structural and functional organization of skeletal tissues in 3D will be crucial to translate the potential of hPSC-derived cells to the clinic. For that purpose, the rapidly-advancing field of biofabrication technologies is likely to offer unprecedented benefits to generate highly complex, patient-specific models. Their integration with advanced bioreactors capable of providing dynamic culture conditions resembling the in vivo micromechanical environment with appropriate stimuli such as compression, tension, and shear, combined with computational modeling will likely facilitate improved methods to scale up the production of large batches of cells. Together, these different advances will reduce costs of cellular therapies, and accelerate the route of hPSC skeletal therapies to the clinic.

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References

[1] Tanaka EM, Reddien PW. The cellular basis for animal regeneration. Dev Cell. 2011;21(1):172–185.
[2] McCusker C, Bryant SV, Gardiner DM. The axolotl limb blastema: cellular and molecular mechanisms driving blastema formation and limb regeneration in tetrapods. Regeneration. 2015;2(2):54–71.
[3] So J, Kim A, Lee SH, et al. Liver progenitor cell-driven liver regeneration. Exp Mol Med. 2020;52(8):1230–1238.
[4] Langer R, Vacanti J. Tissue engineering. Science. 1993;260(5110):920–926.
[5] Wang Y, Yuan M, Guo QY, et al. Mesenchymal stem cells for treating articular cartilage defects and osteoarthritis. Cell Transplant. 2015;24(9):1661–1678.
[6] Amsalem Y, Mardor Y, Feinberg MS, et al. Iron-oxide labeling and outcome of transplanted mesenchymal stem cells in the infarcted myocardium. Circulation. 2007;116(11 Suppl):i38–i45.
[7] Ye J, Bates N, Soteroiu D, et al. High quality clinical grade human embryonic stem cell lines derived from fresh discarded embryos. Stem Cell Res. 2017;8:1–13.
[8] Sharma R, Khristov V, Rising A, et al. Embryonic stem cell-derived retinal pigment epithelium patch rescues retinal degeneration in rodents and pigs. Sci Transl Med. 2019;11(538).
[9] Guo Y, Yu Y, Hu S, et al. The therapeutic potential of mesenchymal stem cells for cardiovascular diseases. Cell Death Dis. 2020;2019;10(1):1–10.
[10] Zhou P, Shi JM, Song JE, et al. Establishing a deeper understanding of the osteogenic differentiation of monolayer cultured human pluripotent stem cells using novel and detailed analyses. Stem Cell Res Ther. 2021;12(1):1.
[11] Kawai S, Yoshitomi H, Sunaga J, et al. In vitro bone-like nodules generated from patient-derived iPSCs recapitulate pathological bone phenotypes. Nat Biomed Eng. 2019;3(7):558–570.
[12] Koay EJ, Hoben GMB, Athanasiou KA. Tissue engineering with chondrogenically differentiated human embryonic stem cells. Stem Cells. 2007;25(9):2183–2190.
[13] Kramer J, Hegert C, Guan K, et al. Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. Mech Dev. 2000;92(2):193–205.
[14] Sottile V, Thomson A, McWhir J. In vitro osteogenic differentiation of human ES cells. Cloning Stem Cells. 2003;5(2):149–155.
[15] Diekman BO, Christoforou N, Willard VP, et al. Cartilage tissue engineering using differentiated and purified induced pluripotent stem cells. Proc Natl Acad Sci USA. 2012;109(47):191172–191177.
[16] Vats A, Biely RC, Tolley N, et al. Chondrogenic differentiation of human embryonic stem cells: the effect of the micro-environment. Tissue Eng. 2006;12(6):1687–1697.
[17] Sui Y, Clarke T, Khillan JS. Limb bud progenitor cells induce differentiation of pluripotent embryonic stem cells into chondrogenic lineage. Differentiation. 2003;71(9–10):578–585.
[18] Nguyen D, Hägg DA, Forsman A, et al. Cartilage tissue engineering by the 3D bioprinting of iPS cells in a nanocellulose/alginate bioink. Sci Rep. 2017;7(1):658–610.
[19] Yi Y, Choi KB, Lim CL, et al. Irradiated human chondrocytes expressing bone morphogenetic protein 2 promote healing of osteoporotic bone fracture in rats. Tissue Eng Part A. 2009;15(10):2853–2863.
[20] Woods S, Bates N, Dunn SL, et al. Generation of human-induced pluripotent stem cells from anterior cruciate ligament. J Orthop Res. 2020;38(1):92–104.
[21] Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. Cytotherapy. 2006;8(4):315–317.
[22] Jeon OH, Panicker LM, Lu Q, et al. Human iPSC-derived osteoblasts and osteoclasts together promote bone regeneration in 3D biomaterials. Sci Rep. 2016;6:26761–26711.
[23] Villa-Diaz LG, Brown SE, Liu Y, et al. Derivation of mesenchymal stem cells from human induced pluripotent stem cells cultured on synthetic substrates. Stem Cells. 2012;30(6):1174–1181.
[24] Teramura T, Onodera Y, Mihara T, et al. Induction of mesenchymal progenitor cells with chondrogenic property from Mouse-Induced pluripotent stem cells. Cell reprogramming (formerly "cloning stem cells"). Cell Reprogram. 2010;12(3):249–261.
[25] Koyama N, Miura M, Nakao K, et al. Human induced pluripotent stem cells differentiated into chondrogenic lineage via generation of mesenchymal progenitor cells. Stem Cells Dev. 2013;22(1):102–113.
[26] Guzzo RM, Gibson J, Xu RH, et al. Efficient differentiation of human iPSC-derived mesenchymal stem cells to chondroprogenitor cells. J Cell Biochem. 2013;114(2):480–490.
[27] Evseenko D, Zhu Y, Schenke-Layland K, et al. Mapping the first stages of mesoderm commitment during differentiation of human embryonic stem cells. Proc Natl Acad Sci USA. 2010;107(31):13742–13747.
[28] De Peppo GM, Marcos-Campos I, Kahler DJ, et al. Engineering bone tissue substitutes from human induced pluripotent stem cells. Proc Natl Acad Sci USA. 2013;110(21):8680–8685.
[29] Limraksasin P, Kondo T, Zhang M, et al. In vitro fabrication of hybrid bone/cartilage complex using mouse induced pluripotent stem cells. Int J Mol Sci. 2020;21(2):581.
[30] Chen JL, Yin Z, Shen WL, et al. Efficacy of hESC-MSCs in knitted silk-collagen scaffold for tendon tissue engineering and their roles. Biomaterials. 2010;31(36):9438–9451.
[31] Rodin S, Domogatskaya A, Ström S, et al. Long-term self-renewal of human pluripotent stem cells on
human recombinant laminin-511. Nat Biotechnol. 2010;28(6):611–615.

[32] Yang YHK, Ogando CR, Wang See C, et al. Changes in phenotype and differentiation potential of human mesenchymal stem cells aging in vitro. Stem Cell Res Ther. 2018;9(1):131–114.

[33] Okita K, Yamakawa T, Matsumura Y, et al. An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. Stem Cells. 2013;31(3):458–466.

[34] Narita M, Yamanaka S, Ichisaka T, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131(5):861–872.

[35] Hynes K, Menicanin D, Mrozik K, et al. Generation of functional mesenchymal stem cells from different induced pluripotent stem cell lines. Stem Cells Dev. 2014;23(10):1084–1096.

[36] Ono N, Ono W, Nagasawa T, et al. A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. Nat Cell Biol. 2014;16(12):1157–1167.

[37] Zhou X, von der Mark K, Henry S, et al. Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. PLOS Genet. 2014;10(12):e1004820.

[38] Aghajanian P, Mohan S. The art of building bone: emerging role of chondrocyte-to-osteoblast transdifferentiation in endochondral ossification. Bone Res. 2018;6:19–19.

[39] Burridge PW, Matsa E, Shukla P, et al. Chemically defined generation of human cardiomyocytes. Nat Methods. 2014;11(8):855–860.

[40] Han L, Chaturvedi P, Kishimoto K, et al. Directed specification and emergence of mesenchymal progenitors in growing bones. Nat Commun. 2020;11(1):4158.

[41] Kishimoto K, Furukawa KT, Luz-Madrigal A, et al. Bidirectional Wnt signaling between endoderm and mesoderm diversification during foregut organogenesis. Nat Commun. 2020;11(1):4158.

[42] Loh KMM, Chen A, Koh PWW, et al. Mapping the pairwise choices leading from pluripotency to human bone, heart, and other mesoderm cell types. Cell. 2016;166(2):451–468.

[43] Umeda K, Oda H, Yan Q, et al. Long-term expandable SOX9+ chondrogenic ectomesenchymal cells from human pluripotent stem cells. Stem Cell Reports. 2015;4(4):712–726.

[44] Nakajima T, Shibata M, Nishio M, et al. Modeling human somite development and fibrodyplasia ossificans progressiva with induced pluripotent stem cells. Dev. 2018;145(16):dev165431.

[45] Adkar SS, Wu CL, Willard VP, et al. Step-Wise chondrogenesis of human induced pluripotent stem cells and purification via a reporter allele generated by CRISPR-Cas9 genome editing. Stem Cells. 2019;37(1):65–76.

[46] Smith CA, Humphreys PA, Naven MA, et al. Directed differentiation of hPSCs through lateral plate mesoderm for generation of articular cartilage progenitors. bioRxiv. 2021. 2021.03.24.436807.

[47] Morigaki Y, Sakakura E, Tsumekawa Y, et al. Self-organized formation of developing appendages from murine pluripotent stem cells. Nat Commun. 2019;10(1):3802–3813.

[48] McCauley LK, Krebsbach PH, James P, et al. Oral and maxillofacial biology and pathology development of the craniofacial skeleton. Front Immunol. 2013;10:1664.

[49] Craft AM, Ahmed N, Rockel JS, et al. Specification of chondrocytes and cartilage tissues from embryonic stem cells. Development. 2013;140(12):2597–2610.

[50] Bradford STJ, Ranghini EJ, Grimiti E, et al. High-throughput screens for agonists of bone morphogenetic protein (BMP) signaling identify potent benzoxazole compounds. J Biol Chem. 2019;294(9):3125–3136.

[51] Genthe JR, Min J, Farmer DM, et al. Ventromorphins: a new class of small molecule activators of the canonical BMP signaling pathway. ACS Chem Biol. 2017;12(9):2436–2447.

[52] Ohba S, Nakajima K, Komiyama Y, et al. A novel osteogenic helioxanthin-derivative acts in a BMP-dependent manner. Biochem Biophys Res Commun. 2007;357(4):854–860.

[53] Aisenbrey EA, Bilussova G, Payne K, et al. Dynamic mechanical loading and growth factors influence chondrogenesis of induced pluripotent mesenchymal progenitor cells in a cartilage-mimetic hydrogel. Biomater Sci. 2019;7(12):5388–5403.

[54] Oldershaw RA, Baxter MA, Lowe ET, et al. Directed differentiation of human embryonic stem cells toward chondrocytes. Nat Biotechnol. 2010;28(11):1187–1194.

[55] Wang T, Nimgkaratana P, Smith CA, et al. Enhanced chondrogenesis from human embryonic stem cells. Stem Cell Res. 2019;39:101497.

[56] Nakagawa T, Lee SY, Reddi AH. Induction of chondrogenesis from human embryonic stem cells without embryo body formation by bone morphogenetic protein 7 and transforming growth factor beta1. Arthritis Rheum. 2009;60(12):3686–3692.

[57] Craft AM, Rockel JS, Nartiss Y, et al. Generation of articular chondrocytes from human pluripotent stem cells. Nat Biotechnol. 2015;33(6):638–645.

[58] Yamashita A, Morikawa M, Yahara Y, et al. Generation of scaffoldless hyaline cartilaginous tissue from human iPSCs. Stem Cell Reps. 2015;4(3):404–418.

[59] Umeda K, Zhao J, Simmons P, et al. Human chondrogenic paraxial mesoderm, directed specification and prospective isolation from pluripotent stem cells. Sci Rep. 2012;2:455.

[60] Diederichs S, Klampfleuthner FAM, Moradi B, et al. Chondral differentiation of induced pluripotent stem cells without progression into the endochondral pathway. Front Cell Dev Biol. 2019;7:1–10.
[62] Kawata M, Mori D, Kanke K, et al. Simple and robust differentiation of human pluripotent stem cells toward chondrocytes by two Small-Molecule Compounds. Stem Cell Reports. 2019;13(3):530–544.

[63] Kanke K, Masaki H, Saito T, et al. Stepwise differentiation of pluripotent stem cells into osteoblasts using four small molecules under serum-free and feeder-free conditions. Stem Cell Reports. 2014;2(6):751–760.

[64] Depeursinge A, Racoeceanu D, lavindrasana J, et al. Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway. Artif Intell Med. 2010;11:ARTMED1118.

[65] Hameed P, Sen D, Manivasagam G. Small molecule-mediated enhanced osteogenesis of human mesenchymal stem cells: a probable alternate for BMP-2. Regen Eng Transl Med. 2020;6(4):407–418.

[66] Solnica-Krezel L, Sepich DS. Gastrulation: making and shaping germ layers. Annu Rev Cell Dev Biol. 2012;28:687–717.

[67] Warga RM, Kimmel CB. Cell movements during epiboly and gastrulation in zebrafish. Development. 1990;108(4):569–580.

[68] Bronner ME, LeDouarin NM. Development and evolution of the neural crest: an overview. Dev Biol. 2012;366(1):2–9.

[69] Lee G, Kim H, Elkabetz Y, et al. Isolation and directed differentiation of human pluripotent stem cells to multipotent neural crest cells. Proc Natl Acad Sci USA. 2013;110(31):12643–12648.

[70] Chan CKF, Lindau P, Jiang W, et al. Clonal precursor of bone, cartilage, and hematopoietic niche stromal cells. Proc Natl Acad Sci USA. 2013;110(31):12643–12648.

[71] Chan CKF, Seo EY, Chen JY, et al. Identification and specification of the mouse skeletal stem cell. Cell. 2015;160(1–2):285–298.

[72] Worthley DL, Churchill M, Compton JT, et al. Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. Cell. 2015;160(1–2):269–284.

[73] Chan CKF, Wood KA, Rowlands CF, Thomas HB, et al. Modelling the developmental spiculoseomal craniofacial disorder Burn-McKeown syndrome using induced pluripotent stem cells. PLoS One. 2020;15(7):e0233582.

[74] Kidwai F, Mui BWH, Arora D, et al. Lineage-specific differentiation of osteogenic progenitors from pluripotent stem cells reveals the FGF1-RUNX2 association in neural crest-derived osteoprogenitors. Stem Cells. 2020;38(9):1107–1123.

[75] Tada S, Era T, Furusawa C, et al. Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development. 2005;132(19):4363–4374.

[76] Levin M. The roles of activin and follistatin signaling in chick gastrulation. Int J Dev Biol. 1998;42(4):553–559.

[77] Fujiwara T, Dehart DB, Sulik KK, et al. Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. Development. 2002;129(20):4685–4696.

[78] Karabagli H, Karabagli P, Ladher RK, et al. Comparison of the expression patterns of several fibroblast growth factors during chick gastrulation and neurulation. Anat Embryol. 2002;205(5–6):365–370.

[79] D’Amour KA, Agulnick AD, Eliazer S, et al. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotechnol. 2005;23(12):1534–1541.

[80] Wu C, Dicks A, Steward N, et al. Single cell transcriptomic analysis of human pluripotent stem cell chondrogenesis. Nat Commun. 2021;12(1):362.

[81] Chan CKF, Liu J, Wang J, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature. 2010;466(7308):829–834.

[82] Wu Y-X, Jing X-Z, Sun Y, et al. CD146+ skeletal stem cells from growth plate exhibit specific chondrogenic differentiation capacity in vitro. Mol Med Rep. 2017;16(6):8019–8028.

[83] Wu L, Blaughermann C, Kyupelyan L, et al. Human developmental chondrogenesis as a basis for engineering chondrocytes from pluripotent stem cells. Stem Cell Reports. 2013;1(6):575–589.

[84] Chan CKF, Gulati GS, Sinha R, et al. Identification of the human skeletal stem cell. Cell. 2018;175(1):43–56.e21.

[85] Ferguson GB, Van Handel B, Bay M, et al. Mapping molecular landmarks of human skeletal ontology and pluripotent stem cell-derived articular chondrocytes. Nat Commun. 2018;9(1).

[86] Qiraqoz Z, Timilsina S, Czarnowski D, et al. Identification of biomarkers indicative of functional skeletal stem cells. Orthod Craniofac Res. 2019;22(Suppl 1):192–198.
Yin L, Yang Z, Wu Y, et al. Label-free separation of mesenchymal stem cell subpopulations with distinct differentiation potencies and paracrine effects. Biomaterials. 2020;240:119881.

Moroni L, Boland T, Burdick JA, et al. Biofabrication: a guide to technology and terminology. Trends Biotechnol. 2018;36(4):384–402.

Tahmasebi A, Enderami SE, Saberi E, et al. MicroRNA-incorporated electropor nanofibers improve osteogenic differentiation of human-induced pluripotent stem cells. J Biomed Mater Res A. 2020;108(2):377–386.

Abazari MF, Soleimanifar F, Amini Faskhodi M, et al. Improved osteogenic differentiation of human induced pluripotent stem cells cultured on polyvinylidene fluoride/collagen/platelet-rich plasma composite nanofibers. J Cell Physiol. 2020;235(2):1155–1164.

Xie J, Peng C, Zhao Q, et al. Osteogenic differentiation and bone regeneration of iPSC-MSCs supported by a biomimetic nanofibrous scaffold. Acta Biomater. 2016;29:365–379.

Marolt D, Campos IM, Bhumiratana S, et al. Engineering bone tissue from human embryonic stem cells. Proc Natl Acad Sci USA. 2012;109(22):8705–8709.

Fonseca AC, Melchels FPW, Ferreira MJS, et al. Emulating human tissues and organs: a bioprinting perspective toward personalized medicine. Chem Rev. 2020;120(19):11128–11139.

Schwab A, Hélay C, Richards RG, et al. Tissue mimetic hyaluronan bioink containing collagen fibers with controlled orientation modulating cell migration and alignment. Mater Today Bio. 2020;7:1–13.

Castillo M, Mouser V, Chen M, et al. Bi-layered micro-fibre reinforced hydrogels for articular cartilage regeneration. Acta Biomater. 2019;95:297–306.

Park JY, Choi J-C, Shim J-H, et al. A comparative study on collagen type I and hyaluronic acid dependent cell behavior for osteochondral tissue bioprinting. Biofabrication. 2014;6(3):035004.

Mammoto A, Mammoto T, Ingber DE. Mechanosensitive mechanisms in transcriptional regulation. J Cell Sci. 2012;125(13):3061–3073.

Malone AMD, Anderson CT, Tummala P, et al. Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism. Proc Natl Acad Sci USA. 2007;104(33):13325–13330.

Hycraft CJ, Serra R. Chapter 11 cilia involvement in patterning and maintenance of the skeleton. Curr Top Dev Biol. 2008;85:303–332.

Musumeci G. The effect of mechanical loading on articular cartilage. J Funct Morphol Kinesiol. 2016;1(2):154–161.

Correia C, Pereira AL, Duarte ARC, et al. Dynamic culturing of cartilage tissue: the significance of hydrostatic pressure. Tissue Eng Part A. 2012;18(19–20):1979–1991.

Zhao YH, Lv X, Liu YL, et al. Hydrostatic pressure promotes the proliferation and osteogenic/chondrogenic differentiation of mesenchymal stem cells: the roles of RhoA and Rac1. Stem Cell Res. 2015;14(3):283–296.
phosphoproteome of human embryonic stem cell differentiation. Sci Signal. 2011;4(164):rs3.

[125] Sze SK, de Kleijn DV, Lai RC, et al. Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. Mol Cell Proteomics. 2007;6(10):1680–1689.

[126] Bodzon-Kulakowska A, Suder P. Imaging mass spectrometry: instrumentation, applications, and combination with other visualization techniques. Mass Spectrom Rev. 2016;35(1):147–169.

[127] Rocha B, Cillero-Pastor B, Eijkel G, et al. Characterization of lipidic markers of chondrogenic differentiation using mass spectrometry imaging. Proteomics. 2015;15(4):702–713.

[128] Sun AX, Lin H, Sun AX, et al. Acceleration of chondrogenic differentiation of human mesenchymal stem cells by sustained growth factor release in 3D graphene oxide incorporated hydrogels. Acta Biomater. 2020;105:44–55.

[129] Sun AX, Lin H, Fritch MR, et al. Chondrogenesis of human bone marrow mesenchymal stem cells in 3-dimensional, photocrosslinked hydrogel constructs: effect of cell seeding density and material stiffness. Acta Biomater. 2017;58:302–311.

[130] Deng Y, Sun AX, Overholt KJ, et al. Enhancing chondrogenesis and mechanical strength retention in physiologically relevant hydrogels with incorporation of hyaluronic acid and direct loading of TGF-β. Acta Biomater. 2019;83:167–176.

[131] Xiong Z. Fabrication of porous scaffolds for bone tissue engineering via low-temperature deposition. Scr Mater. 2002;46(11):771–776.

[132] Sun AX, Lin H, Beck AM, et al. Projection stereolithographic fabrication of human adipose stem cell-incorporated biodegradable scaffolds for cartilage tissue engineering. Front Bioeng Biotechnol. 2015;3:1–9.

[133] Andrew CD, Susan EC, Emily MR, et al. A comparison of different bioinks for 3D bioprinting of fibrocartilage and hyaline cartilage. Biofabrication. 2016;8:45002.

[134] Zhou M, Lozano N, Wychowaniec JK, et al. Graphene oxide: a growth factor delivery carrier to enhance chondrogenic differentiation of human mesenchymal stem cells in 3D hydrogels. Acta Biomater. 2019;96:271–280.

[135] Campbell K, Naire S, Kuiper JH. A mathematical model of cartilage regeneration after chondrocyte and stem cell implantation – I: the effects of growth factors. J Tissue Eng. 2019;10:2041731419827791.

[136] Campbell K, Naire S, Kuiper JH. A mathematical model of cartilage regeneration after chondrocyte and stem cell implantation – II: the effects of co-implantation. J Tissue Eng. 2019;10:2041731419827792.

[137] Gaspari E, Franke A, Robles-Diaz D, et al. Paracrine mechanisms in early differentiation of human pluripotent stem cells: insights from a mathematical model. Stem Cell Res. 2018;32:1–7.

[138] Libby ARG, Briers D, Haghighi I, et al. Automated design of pluripotent stem cell self-organization. Cell Syst. 2019;9(5):483–495.e10.

[139] Manstein F, Ullmann K, Kropp C, et al. High density bioprocessing of human pluripotent stem cells by metabolic control and in silico modeling. Stem Cells Transl Med. 2021;10(7):1063–0453. sctm.

[140] Wang Z, Wang Z, Lu WW, et al. Novel biomaterial strategies for controlled growth factor delivery for biomedical applications. NPG Asia Mater. 2017;9(10):e435–17.

[141] Kaneko H, Arakawa T, Mano H, et al. Direct stimulation of osteoelastic bone resorption by bone morphogenetic protein (BMP)-2 and expression of BMP receptors in mature osteoclasts. Bone. 2000;27(4):479–486.

[142] Zara JN, Siu RK, Zhang X, et al. High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. Tissue Eng Part A. 2011;17(9–10):1389–1399.

[143] Nimni ME. Polypeptide growth factors: targeted delivery systems. Biomaterials. 1997;18(18):1201–1225.

[144] Yao Q, Jing J, Zeng Q, et al. Bilayered BMP2 eluting coatings on graphene foam by electrophoretic deposition: electroresponsive BMP2 release and enhancement of osteogenic differentiation. ACS Appl Mater Interfaces. 2017;9(46):39962–39970.

[145] Crescente-Campo J, Borrajo E, Vidal A, et al. New scaffolds encapsulating TGF-β3/BMP-7 combinations driving strong chondrogenic differentiation. Eur J Pharm Biopharm. 2017;114:69–78.

[146] De la Riva B, Sánchez E, Hernández A, et al. Local controlled release of VEGF and PDGF from a combined brushite-chitosan system enhances bone regeneration. J Control Release. 2010;143(1):45–52.

[147] Solorio L, Zwolinski C, Lund AW, et al. Gelatin microspheres crosslinked with genipin for local delivery of growth factors. J Tissue Eng Regen Med. 2010;4(7):514–523.

[148] Meinel L, Zoidis E, Zapf J, et al. Localized insulin-like growth factor I delivery to enhance new bone formation. Bone. 2003;33(4):660–672.

[149] Lee CH, Rodeo SA, Fortier LA, et al. Protein-releasing polymeric scaffolds induce fibrochondrocytic differentiation of endogenous cells for knee meniscus regeneration in sheep. Sci Transl Med. 2014;6(266):266ra171.

[150] Nandi SK, Kundu B, Basu D. Protein growth factors loaded highly porous chitosan scaffold: a comparison of bone healing properties. Mater Sci Eng C Mater Biomol Appl. 2013;33(3):1267–1275.

[151] Freeman FE, Kelly DJ. Tuning alginate bioink stiffness driving strong chondrogenic differentiation. Eur J Clin Investig. 2011;41:1275–1279.

[152] Crecente-Campo J, Borrajo E, Vidal A, et al. New scaffolds encapsulating TGF-β3/BMP-7 combinations driving strong chondrogenic differentiation. Eur J Pharm Biopharm. 2017;114:69–78.

[153] Lee CH, Rodeo SA, Fortier LA, et al. Protein-releasing polymeric scaffolds induce fibrochondrocytic differentiation of endogenous cells for knee meniscus regeneration in sheep. Sci Transl Med. 2014;6(266):266ra171.
complexes under magnetic field conditions. J Biomed Mater Res A. 2010;92(1):196–204.

[154] Solorio LD, Dhami CD, Dang PN, et al. Spatiotemporal regulation of chondrogenic differentiation with controlled delivery of transforming growth factor-β1 from gelatin microspheres in mesenchymal stem cell aggregates. Stem Cells Transl Med. 2012;1(8):632–639.

[155] Fenno L, Yizhar O, Deisseroth K. The development and application of optogenetics. Annu Rev Neurosci. 2011;34:389–412.

[156] Krueger D, Izquierdo E, Viswanathan R, et al. Principles and applications of optogenetics in developmental biology. Development. 2019;146(20):dev175067.

[157] Leopold AV, Chernov KG, Verkhusha VV. Optogenetically controlled protein kinases for regulation of cellular signaling. Chem Soc Rev. 2018;47(7):2454–2484.

[158] Mumford TR, Roth L, Bugaj LJ. Reverse and forward engineering multicellular structures with optogenetics. Curr Opin Biomed Eng. 2020;16:61–71.

[159] Repina NA, Rosenbloom A, Mukherjee A, et al. At light speed: advances in optogenetic systems for regulating cell signaling and behavior. Annu Rev Chem Biomol Eng. 2017;8:13–39.

[160] Tischer D, Weiner OD. Illuminating cell signalling with optogenetic tools. Nat Rev Mol Cell Biol. 2014;15:551–558.

[161] Hu W, Li Q, Li B, et al. Optogenetics sheds new light on tissue engineering and regenerative medicine. Biomaterials. 2020;227:119546.

[162] Spagnuolo G, Genovese F, Fortunato L, et al. The impact of optogenetics on regenerative medicine. Appl Sci. 2020;10(7):173.

[163] Sako K, Pradhan SJ, Barone V, et al. Optogenetic control of nodal signaling reveals a temporal pattern of nodal signaling regulating cell fate specification during gastrulation. Cell Rep. 2016;16(3):866–877.

[164] Rogers KW, Elgamacy M, Jordan BM. Optogenetic investigation of BMP target gene expression diversity. Elife. 2020;9:1–44.

[165] Li Y, Lee M, Kim N, et al. Spatiotemporal control of TGF-β signaling with light. ACS Synth Biol. 2018;7(2):443–451.

[166] Humphreys PA, Woods S, Smith CA, et al. Optogenetic control of the BMP signaling pathway. ACS Synth Biol. 2020;9(11):3067–3078.

[167] Wang W, Huang D, Ren J, et al. Optogenetic control of mesenchymal cell fate towards precise bone regeneration. Theranostics. 2019;9(26):8196–8205.

[168] Aly RM. Current state of stem cell-based therapies: an overview. Stem Cell Investig. 2020;7:8–10.

[169] Mancuso P, Raman S, Glynn A, et al. Mesenchymal stem cell therapy for osteoarthritis: the critical role of the cell secretome. Front Bioeng Biotechnol. 2019;7:9.