



## Review

## Developmental principles informing human pluripotent stem cell differentiation to cartilage and bone



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## ABSTRACT

Human pluripotent stem cells can differentiate into any cell type given appropriate signals and hence have been used to research early human development of many tissues and diseases. Here, we review the major biological factors that regulate cartilage and bone development through the three main routes of neural crest, lateral plate mesoderm and paraxial mesoderm. We examine how these routes have been used in differentiation protocols that replicate skeletal development using human pluripotent stem cells and how these methods have been refined and improved over time. Finally, we discuss how pluripotent stem cells can be employed to understand human skeletal genetic diseases with a developmental origin and phenotype, and how developmental protocols have been applied to gain a better understanding of these conditions.

## 1. Introduction

Skeletal development is a complex and highly orchestrated process in which a number of coordinated molecular signalling networks regulate skeletal tissue specification [1]. In preceding decades, scientists have utilised mouse genetics, transgenics and vertebrate embryology to great effect, providing tremendous strides in understanding how skeletal elements develop and are subsequently maintained. Concurrently, stem cells that can be expanded almost indefinitely in culture, while retaining the ability to differentiate into any cell type of the body (pluripotent stem cells), have appeared particularly attractive as a tool to investigate developmental processes. Animal models, especially transgenic mice, allow investigation of normal and aberrant mouse development, but this experimental route is not available for understanding human development. Furthermore, there is great incentive to recapitulate human skeletal tissue generation, because of the inability of damaged or diseased cartilage and (some) bone fractures to adequately heal [2]. This has prompted attempts at generating cellular repair and replacement

through tissue engineering strategies, often starting with stem cells [3].

At the end of the 20th century, Thomson and colleagues [4] demonstrated that human embryonic stem cells (hESCs) could be generated from human blastocyst stage embryos, in a similar manner to that already established in mice [4–6]. The isolation and expansion of hESCs revolutionised the field of human developmental biology and opened the door for in vitro generation of various tissue types for study and therapeutic applications. Further progress was made with the generation of induced pluripotent stem cells (iPSCs) from human somatic cells [7,8], with expansion and differentiation properties similar to those of hESCs. Currently, human pluripotent stem cells (hPSCs) are globally available to research groups, including those interested in skeletogenesis. Within the skeletal research community, the focus has been upon development of hPSC differentiation protocols for chondrocytes and osteoblasts, with fewer available for tendon and ligament. There is also a considerable body of literature on hPSC-skeletal muscle [9] and some on intervertebral disc [10] differentiation. This review covers recent progress in the generation of bone and cartilage from hPSCs, and

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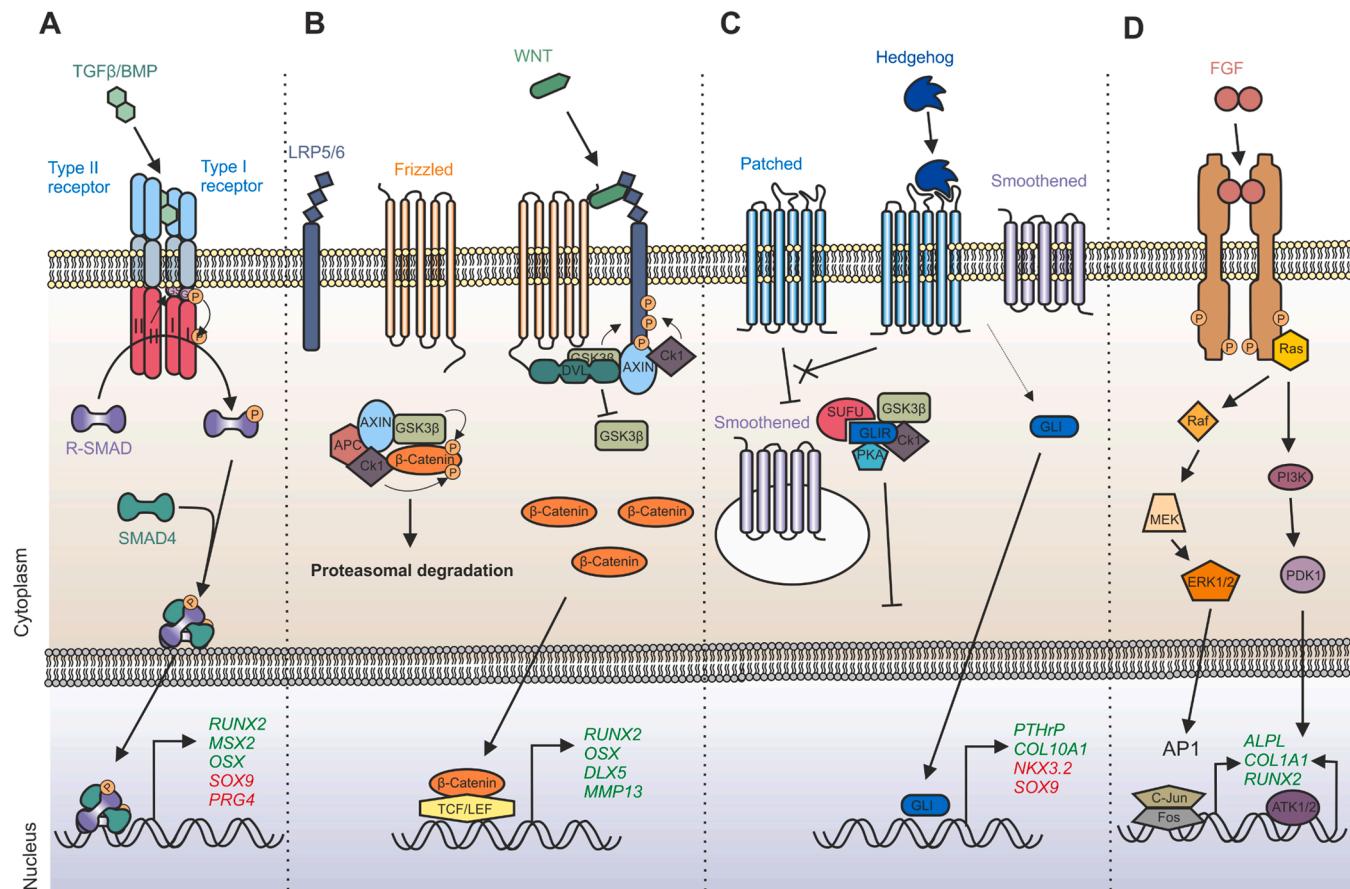
how lessons from development have informed hPSC differentiation methodology. We set out to illustrate how the past decades of developmental findings, such as recognising early stage-specific factors in regional mesoderm formation and induction of different skeletal subtypes, has improved protocols aimed at generating lineage-specific authentic chondrogenic and osteogenic cells. Such protocols facilitate analysis of both healthy and aberrant human skeletal development, enable drug discovery or safety screens and finally, provide cells for use in therapeutic tissue repair.

## 2. Key developmental principles for bone and cartilage formation

Bone and cartilage arise from several distinct embryonic lineages, and their developmental origin is reflected in extracellular matrix (ECM) composition, structure and anatomical location. Craniofacial cartilage and bones of the cranial vault are primarily derived from the ectodermal neural crest [11], axial skeletal components from paraxial mesoderm [12] and the appendicular skeleton from lateral plate mesoderm [13]. The molecular regulators and mechanisms responsible for bone and

cartilage formation are often conserved across embryonic lineages, resulting in chondrogenic and osteogenic cells that share common progenitors and several transcriptional and phenotypic characteristics.

Bone is established during embryogenesis through either intramembranous or endochondral ossification (reviewed [14]). Flat bones, including the cranial vault (calvaria) bones of the skull and maxilla, are formed through intramembranous ossification, in which mesenchymal cells directly differentiate into mineralising osteoblasts. By contrast, during endochondral ossification, chondrocytes form a transient cartilage scaffold which is then remodelled into bone (Section 2.3). Permanent populations of phenotypically stable chondrocytes also persist into adulthood, such as those found in the nasal septum, costal rib extensions and in articular joint cartilage [13]. The generation of these permanent chondrocytes is of particular interest for skeletal tissue engineering due to the poor regenerative capacity of adult cartilage. However, the mechanisms which delineate chondrocyte fates are currently unclear. Furthermore, signalling crosstalk, cellular microenvironment and embryonic origin can affect the matrix components that chondrocytes and osteoblasts synthesise. Therefore, it is critical to consider the developmental routes to chondrogenesis and osteogenesis



**Fig. 1. Schematic of key signalling pathways responsible for bone and cartilage formation.** (A) **Canonical TGF $\beta$ /BMP signalling.** TGF $\beta$  and BMP ligands bind to either a Type I or II receptor forming a heterotetrameric signalling complex of two Type I and two Type II receptors. The constitutively active Type II receptor transphosphorylates the Type I, leading to activation of Type I kinase and recruitment of the R-SMAD intracellular mediators (SMAD2/3 for TGF $\beta$  and SMAD1/5/8 for BMP). Phosphorylated R-SMAD proteins form a heterotrimeric complex with the mediator SMAD (SMAD4) then translocate and accumulate within the nucleus to elicit transcriptional changes. (B) **Canonical WNT signalling.** In the absence of WNT ligand, the degradation complex of APC/Axin/CKI $\alpha$ /GSK3 $\beta$  binds to  $\beta$ -catenin and targets it for degradation. Upon binding of a WNT ligand to Frizzled (FZ) and subsequent recruitment of LRP5/6 co-receptor, activation of DVL prevents APC/Axin/CKI $\alpha$ /GSK3 $\beta$  from binding to  $\beta$ -catenin, thus enabling its stabilisation, nuclear translocation and activation of WNT-target genes with TCF/LEF proteins. (C) **Hedgehog signalling.** In the absence of ligand, Patched (PTCH) inhibits Smoothened (SMO) activity, preventing it from entering the primary cilia. Inhibition of SMO results in degradation of transcriptional activator Gli via binding within a degradation complex consisting of SUFU, GSK3 $\beta$ , PKA and CK1. Binding of HH ligands to PTCH relieves its inhibitory function, enabling SMO to enter the primary cilia and activate Gli, which translocates to the nucleus and initiates transcription. (D) **FGF signalling.** Binding of FGF ligands to FGFRs with the co-factor heparan sulphate enables activation of intracellular FGFR tyrosine kinase residues. Active FGFRs are coupled with a variety of intracellular signalling including Ras-MAPK, PI3K-AKT PLC $\gamma$ , and STAT. Examples of target gene expression induced by each pathway highlighted in green (more relevant to osteogenesis) and red (more relevant to chondrogenesis).

when deciding upon hPSC differentiation approaches.

## 2.1. Key molecular regulators of bone and cartilage formation

### 2.1.1. TGF $\beta$ and BMP signalling family

The transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily of signalling molecules, which includes TGF $\beta$ s and bone morphogenetic proteins (BMPs), is a group of structurally-related proteins that play a prominent role in both cartilage and bone formation across embryonic lineages [15, 16]. There are over 30 distinct TGF $\beta$ -family ligands which are broadly grouped as either TGF $\beta$ -like, including TGF $\beta$ 1–3, Activin and Nodal, or BMP-like, including BMP2,4,6,8,10, growth differentiation factor (GDF) 5,6 and anti müllerian hormone (AMH) depending upon sequence similarity and their downstream effects [17]. All TGF $\beta$ -superfamily ligands interact with the extracellular ligand-binding domain of transmembrane serine/threonine receptor kinases which comprise 12 members: 7 Type-I and 5 Type-II receptors [18]. Signal transduction requires the formation of a heterotetrameric protein complex consisting of two Type I and two Type II receptors [19]. During canonical signalling, active receptor complexes facilitate phosphorylation of the SMAD transcriptional regulators, either SMAD2/3 in TGF $\beta$  signalling or SMAD1/5/8 in BMP signalling (Fig. 1 A) [20].

### 2.1.2. WNT signalling

The wingless-related integration site (WNT) signalling family of molecules encompasses a range of glycoprotein ligands, including WNT1,3,5A,6, and WNT7A [21]. Canonical WNT signalling is  $\beta$ -catenin-dependent, executed through its intracellular accumulation followed by nuclear translocation and subsequent regulation of target gene expression (Fig. 1B). In the absence of WNT ligands,  $\beta$ -catenin is bound within a complex consisting of glycogen synthase kinase 3  $\beta$  (GSK3 $\beta$ ), casein kinase 1 (CK1), axin and adenomatous polyposis coli (APC) [22]. Within this complex, the N-terminal degradation domain of  $\beta$ -catenin is phosphorylated by GSK3 $\beta$  and CK1, targeting it for degradation. Signal transduction occurs when WNT ligands bind to Frizzled (FZ) receptors and low-density lipoprotein receptor-related protein (LRP) co-receptors. Ligand binding results in recruitment and activation of Dishevelled (DVL), which inhibits GSK3 $\beta$  and thus disrupts formation of the degradation complex [23].  $\beta$ -catenin then accumulates within the cytoplasm, translocates to the nucleus and associates with T cell factor/lymphoid enhancer factors (TCF/LEF) for transcriptional activation. Non-canonical  $\beta$ -catenin-independent WNT signalling can occur via the calcium or planar cell polarity (PCP) pathways [24–27].

### 2.1.3. HH signalling

The hedgehog (HH) signalling pathway is highly conserved [28], and plays important roles during skeletal development [29,30]. In mammals, it is activated by three different HH ligands; sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH) [31]. The HH ligands require proper processing by the secreting cell, including C-terminal cholesterol modification and N-terminal palmitoylation, which affect the morphogen gradient produced. HH signal transduction includes the patched (PTCH) receptor, smoothened (SMO) signal transducer and GLI transcription factors [32–35]. In the absence of an HH ligand, PTCH inhibits SMO. Upon binding of an HH ligand to PTCH, the inhibitory effect on SMO is lost, SMO then accumulates within primary cilia initiating activation of GLI family members (Fig. 1 C) [35].

### 2.1.4. FGF signalling

Fibroblast growth factors (FGFs) comprise a family of 23 members which interact with FGF receptor (FGFR) monomers. In conjunction with heparan sulphate for the canonical ligands FGF1–10, binding induces dimerization and tyrosine kinase transactivation, including that of adapter proteins (Fig. 1D) [36,37]. FGFs are heavily and complexly involved in most aspects of cartilage and bone development as highlighted below. The most critical FGFs for bone and cartilage formation

are FGF1,2,4,8,9 and 10 (canonical group) and 18,23 (endocrine group), which transduce activity through FGFRs 1–3.

## 2.2. Embryonic routes to bone and cartilage

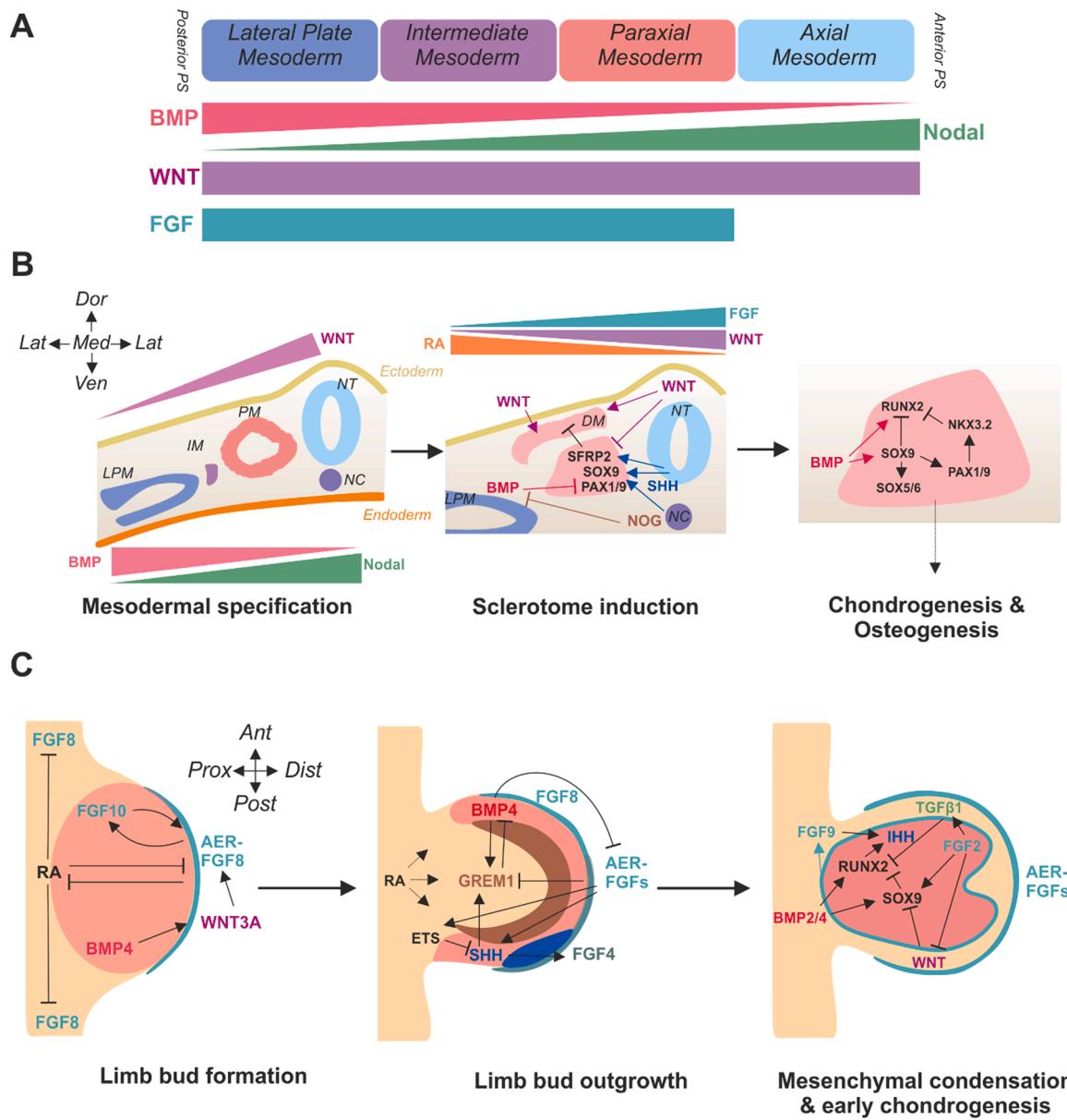
### 2.2.1. Ectodermal neural crest

Neural crest cells (NCCs) are a multipotent population of post-gastrulation progenitor cells established during early neurulation. NCCs undergo epithelial-mesenchymal transition (EMT) and delaminate from the ectodermal neuroepithelium [38], migrating to a multitude of embryonic locations and contributing to different morphogenetic processes depending upon their anterior-posterior axial location [39]. NCC patterning is primarily controlled by *Homeobox (Hox)* and *Distal-less (Dlx)* family transcription factors, which commit NCCs into region-specific categories including cranial, trunk, cardiac and vagal [40]. Certain skeletal components of the head and neck, particularly the ventral portions, are derived from cranial NCCs. Other contributions of cranial NCCs include nervous, muscular and connective tissues [41]. In mice and chicks, cranial NCC derivatives include the frontal, nasal, premaxilla, squamosal, and dentary regions [42]. These diverge through expression, or lack of expression, of various anterior *Hox* genes [43]: *Hox* gene expression is associated with cells that colonise the second pharyngeal arch, whilst cells that lack *Hox* gene expression populate the first pharyngeal arch and subsequently establish skeletal tissue including Meckel's cartilage, the precursor to the lower jaw, and the maxillae. For example, HOX-A2 inhibits expression of *SRY-box transcription factor 9 (Sox9)*, which would otherwise drive ectomesenchymal specification, marked by expression of *Dlx2* and *Dlx5* [44–46].

BMP signals drive expression of *Dlx2/Dlx5*, *Sox9* and *Runt-related transcription factor 2 (Runx2)*, facilitating ectomesenchymal condensation and subsequent formation of an osteochondral progenitor population [44–46]. Most cranial bones are then formed through endochondral ossification (see Section 2.3 ), although the bones of the calvaria are formed through intramembranous ossification [47]. FGF2 stimulates *Bmp2* expression in osteoblasts during cranial development, which also acts synergistically with other factors during bone formation through a series of complex positive interactions, including inhibition of the BMP antagonist Noggin [48,49]. Mutation of FGFR2 can cause features similar to Apert syndrome, characterized by premature cranial suture closure, distinct facial characteristics and hand and foot anomalies [50]. FGF2 also induces maturation of osteoblasts through stimulation of WNT/ $\beta$ -catenin signalling [51]. Development of the cranial neural crest (CNC)-derived skeleton is impaired in  $\beta$ -catenin mutants, with defects in most bone and cartilage [52]. Interestingly, the role of canonical WNT signalling in CNC-cartilage development appears to be context-dependent, with recent work suggesting that enhanced  $\beta$ -catenin levels in CNC cells, due to increased BMP-SMAD signalling, can lead to ectopic chondrogenesis [53].

### 2.2.2. Paraxial mesoderm

Most components of the axial skeleton, including the ribs and vertebrae, are formed from paraxial mesoderm [12]. Mesodermal specification occurs during induction of the primitive streak (PS) [54, 55], which establishes the site of gastrulation and embryonic bilateral symmetry. Initially, BMP signals derived from the trophectoderm, interacting with TGF $\beta$  (Nodal) and WNT signalling in the epiblast, are critical for establishing the PS [56–58]. During gastrulation, mesodermal derivatives become fated to form different cell types along the mediolateral axis as they migrate through the PS (Fig. 2A). Anterior PS cells form axial mesoderm (notochord), which is flanked by the other mesodermal subtypes. From medial to lateral, mid-posterior PS cells form paraxial mesoderm, followed by intermediate mesoderm (IM), whilst the most posterior PS cells form lateral plate mesoderm (LPM). Mesodermal subtypes are specified by opposing directional cues from BMP and TGF $\beta$  (Nodal) [59], with high Nodal signals inducing anterior PS [57] and high BMP inducing posterior PS fates [60]. WNT signalling



**Fig. 2. Overview of early developmental processes leading to cartilage and bone.** (A) Summary of signals that control mesodermal specification in the primitive streak (PS) (B) Schematic of signalling networks responsible for specification of the sclerotome. **Left.** Mesoderm specification and embryonic orientation are regulated by an interplay of morphogenetic signalling gradients. **Middle.** Paraxial mesoderm (PM) is specified into the dermomyotome (DM), giving rise to trunk and limb musculature, and sclerotome, giving rise to bone and cartilage. SHH signals from the notochord and floor plate of the neural tube (NT) induce sclerotome. SHH and neural WNT compete and a gradient of Noggin from the notochord antagonizes lateral plate mesoderm-derived BMP signals. **Right.** BMP signals drive chondrogenic differentiation of the mesenchymal sclerotome. (C) Schematic of the signalling networks responsible for early appendicular skeletal development. **Left.** Dorso-ventral polarisation and establishment of AER with mutual antagonism between RA and FGF8 ensures correct orientation of the limb field. AER is induced through BMP from the mesenchyme and ectodermal WNT. **Middle.** Patterning and outgrowth of the limb bud mesenchyme is driven by the ZPA SHH signalling center. Early BMP signals upregulate *Grem1*, enhanced by SHH, resulting in low BMP activity during establishment of the SHH/AER-FGF/Gremlin-1 feedback loop. Outgrowth ends with termination of this feedback loop through FGF inhibition of Gremlin-1 and high ETS which inhibits SHH. **Right.** Mesenchymal condensation is regulated by re-emerging BMP with TGF $\beta$  and FGF signals from the peripheral mesenchyme. Inhibitory WNT signals contribute to the formation of the perichondrium to define the skeletal border during endochondral ossification.

Adapted from [1,74,93,94].

is also crucial for early paraxial mesoderm development, being required for mid-PS fates [61] and inducing expression of transcriptional regulators including *Brachyury* (*T*), *T-box transcription factor 6* (*Tbx6*) and *Mesogenin1* (*Msgn1*) [62–64]. FGF signalling through FGFR1 additionally regulates EMT in the PS at gastrulation and mesoderm formation [55]. High BMP signalling from the LPM then promotes lateralisation of mesoderm [65]. BMP4 is thought to be the primary BMP family member responsible for mesodermal patterning in the PS in tandem with

region-specific concentrations of Nodal (Fig. 2B, Left) [60,66]. Activity of the BMP inhibitor Noggin, from the adjacent notochord, prevents lateralisation of the pre-somitic mesoderm and establishes a BMP-gradient during mesodermal patterning [67,68].

Paraxial mesoderm undergoes somitogenesis through cyclical morphogenetic signals controlled by the segmentation clock, regulated by FGF and NOTCH signalling [69–72]. Somite formation in mice is regulated by FGF8 and WNT in a posterior-to-anterior gradient,

established by an mRNA decay mechanism, with a retinoic acid gradient in the opposite direction [73]. FGF signals act to control the pace of segmentation in opposite phase to WNT/β-catenin. Mature somites are divided into two primary populations, the sclerotome at the ventromedial side and the dermomyotome at the epithelial dorsolateral side [74]. In mice, sclerotome induction relies upon SHH signals from notochord and neural tube cells [75], which induce expression of sclerotome markers *Pax1*, *Nkx3.2* and the chondrogenic regular *Sox9* (Fig. 2B, Middle) [76,77]. Sclerotome specification initially requires antagonism of BMP by continued notochordal Noggin activity [78], but later chondrogenesis is maintained by BMP signalling crosstalk with SOX9 and NKX3–2 [76]. The chondrogenic role of BMP signals requires initial SHH activity to upregulate *Nkx3.2* and *Sox9* [76,79] but conversely, SHH signalling cannot induce chondrogenesis alone, and requires modulation by BMP (Fig. 2B, Right) [80]. Skeletal components, including cartilage and bone of the vertebrae and most of the ribs, are derived from the sclerotome whilst the dermomyotome primarily gives rise to trunk and limb muscle in addition to dermis [81]. Besides its role for axial skeletal tissue development, the paraxial mesoderm also contributes to craniofacial skeletal elements, particularly the parietal, occipital and sphenoid regions in mice, and similar posterior regions in chick and fish [42].

### 2.2.3. Lateral plate mesoderm and limb mesenchyme

Lateral plate mesoderm is specified by a gradient of BMP signals in the PS [68,82,83], with high BMP critical in LPM specification. Bone and cartilage of the limbs are derived from limb bud mesenchyme, which itself arises from somatic LPM. The first overt sign of limb morphogenesis is the formation of the limb bud: a protruding structure consisting of migrating mesenchymal cells within an ectoderm-covered evagination [1]. Limb patterning requires precise spatiotemporal cues driven by the signalling centers of the apical ectodermal ridge (AER), the zone of polarizing activity (ZPA) and the non-AER limb ectoderm [84]. Limb patterning and outgrowth is mediated by the AER, with various members of the FGF family of signalling proteins essential in a time- and dose-dependent fashion [85]. An antagonistic feedback loop is established between proximal retinoic acid (RA) cues, AER-FGF8 and non-AER ectoderm-derived WNT3A that, alongside BMP signals, drive limb bud outgrowth (Fig. 2B, Left). Throughout limb bud development, an undifferentiated population of mesenchymal cells is maintained in its center, where synergistic activity of WNT and FGF maintain it in a proliferative and multipotent state [86]. BMP signalling is restricted to the distal edge of the limb bud by Gremlin stimulated by the ZPA SHH signalling center, which additionally drives FGF from the AER, generating a SHH/AER-FGF/Gremlin-1 feedback loop [87,88] (Fig. 2B, Middle). FGFR1 deletion prior to mesenchymal condensation leads to malformation of the AER and absence of distal limb (autopod), while later inactivation affects only the first 2 digits, where it has been suggested that it is redundant with FGFR2 [89,90].

When the SHH/AER-FGF/Gremlin-1 feedback loop is removed, through inhibition of *Grem1* by AER-FGFs and termination of the SHH signalling center, high BMP activity promotes the transition of mesenchymal progenitor cells into proliferating pre-chondrocytes that undergo endochondral ossification (Fig. 2B, Right). Lineage tracing in mice indicates that cells positive for paired mesoderm homeobox protein 1 (*Prrx1*) mark the common progenitor population for almost all of the connective tissue cell types in the appendicular skeleton [91]. Additionally, a recent single-cell transcriptomic study identified a specific subset of human limb bud mesenchymal cells, positive for *PRRX1* and the master chondrogenic regulator *SOX9*, which give rise to osteochondral progenitors [92].

## 2.3. Molecular regulation of endochondral ossification

### 2.3.1. Mesenchymal condensation

Bone is formed through the mechanisms of intramembranous ossification (Fig. 3A) or, more commonly, endochondral ossification during

development of the appendicular skeleton (Fig. 3B). Mesenchymal cell condensation initiates endochondral ossification, firstly through the formation of a continuous hyaline cartilage anlage rich in type II collagen and aggrecan [1]. Chondrogenesis is actively controlled by time-dependent BMP signalling, particularly BMP2 and BMP4, which promotes condensation of mesenchymal cells and the expression of regulatory transcription factors *Sox9*, *Sox5* and *Sox6*, stimulating synthesis of cartilage-related ECM proteins [95–98] (Fig. 3B). Mesenchymal cells surrounding the anlage form the perichondrium, defining the border of the embryonic skeleton. WNT induces methylation and inhibition of the *Sox9* promoter [99], opposed by FGF signalling from the apical ectodermal ridge, which allows the condensing mesenchyme to differentiate to chondrocytes.

### 2.3.2. Establishment of the primary and secondary ossification centers

Cells within the center of the anlage undergo rapid maturation driven by canonical BMP signalling, resulting in upregulation of the hypertrophic regulator *Runx2* and downstream markers including *COL10A1* and vascular endothelial growth factor (VEGF) [100–102]. VEGF leads to vascular invasion of the anlage, resulting in an influx of osterix (*Sp7*) positive osteoblastic precursors from the perichondrium and the haematopoietic system [103]. These form osteocalcin (BGLAP) positive osteoblasts that contribute to bone formation [104].

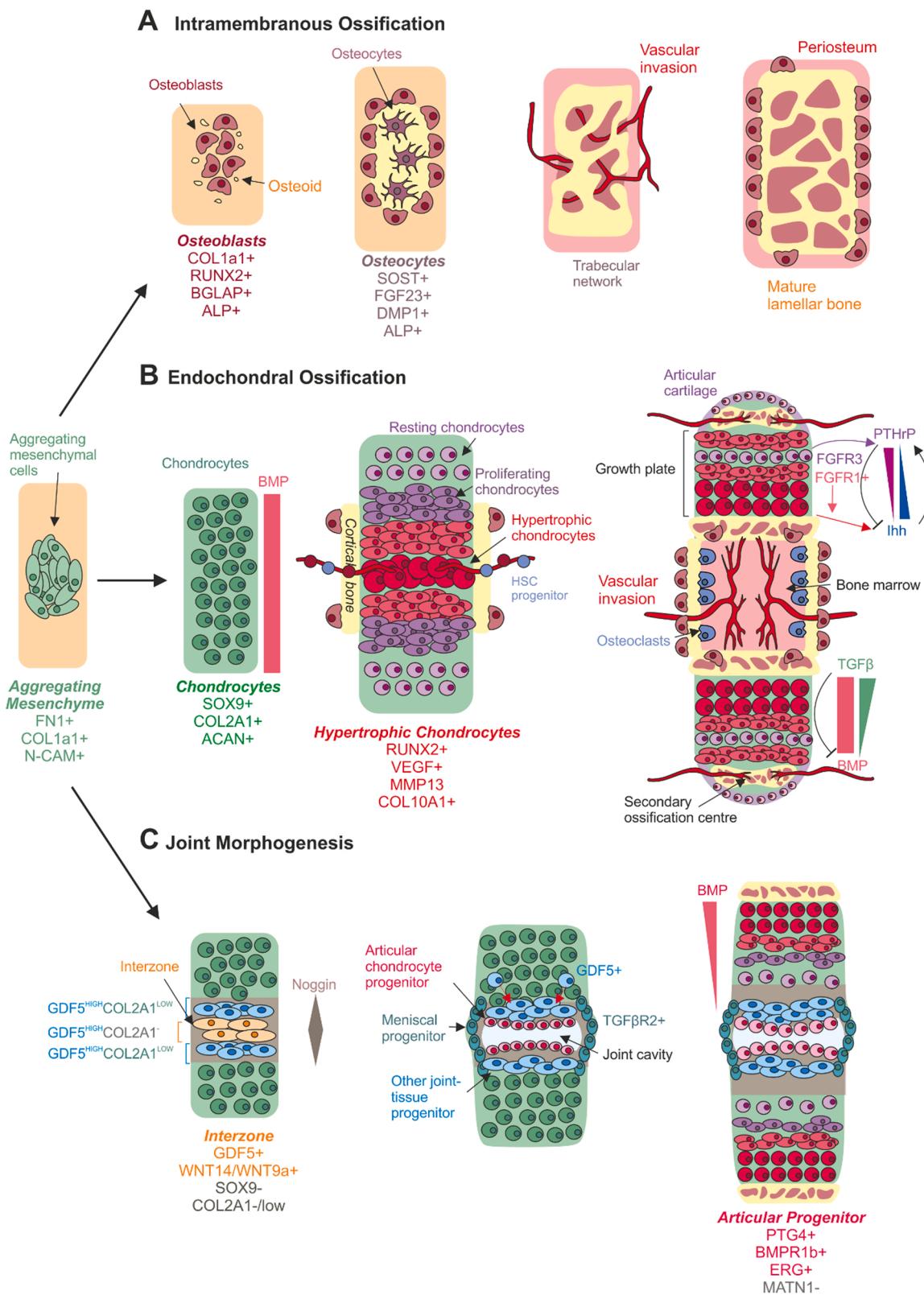
Vascular invasion and excavation of the early cartilage matrix by osteoclasts results in the bisection of the endochondral structure and establishes the primary ossification center where osteoblasts begin to lay down bone matrix [105]. Chondrocytes in the growth plate continue to undergo proliferation and hypertrophy to lengthen the bone. The secondary ossification centers are formed when chondrocytes within the epiphysis also undergo hypertrophy, leading to vascularisation and mineralisation, forming two distally distinct cartilaginous epiphyseal growth plates (Fig. 3B, right).

### 2.3.3. Growth plate signalling

Growth plate cartilage is composed of stratified phenotypically distinct zones leading to controlled ossification of chondrocytes in a continuous fashion, driving longitudinal bone growth that continues beyond birth. From the distal end, the structure of the growth plate is as follows: the reserve zone, proliferative zone, pre-hypertrophic and hypertrophic zones, calcification zone and finally the zone of ossification (Fig. 3B).

Chondrocyte differentiation is modulated by a negative signalling feedback loop consisting of IHH and parathyroid hormone-related peptide (PTHRP) with additional crosstalk between FGF and BMP [106,107]. IHH drives chondrocytes towards hypertrophy and promotes osteogenesis through downstream inhibition of GLI family members [108]. However, IHH signals result in the expression of PTHRP, which inhibits the hypertrophic activity of IHH and RUNX2 whilst additionally promoting chondrocyte proliferation through Cyclin D1 activity [109–111]. Deletion of *Ihh* in mice results in premature chondrocyte hypertrophy, due to the lack of PTHRP synthesis, and bone growth abnormalities, likely as a result of depleted osteoblasts within the primary spongiosa [112]. PTHRP is also antagonized by WNT-stimulated β-catenin, which drives chondrocyte hypertrophy and promotes osteogenesis [113]. Deletion of β-catenin in mouse head and limb mesenchyme results in impaired osteoblast differentiation and a shift towards chondrogenesis [114,115]. The non-canonical β-catenin-independent PCP pathway is responsible for orientating chondrocyte cell division in the growth plate [27].

Formation of the growth plate requires expression of FGFR3 by immature chondrocytes under SOX9 regulation [116]. Indeed, FGFR3 tyrosine kinase domain mutations cause chondrodysplasia in sheep [117]. In proliferating chondrocytes, FGF-stimulated pro-chondrogenic effects occur through the Ras/Raf-MAPK pathway, inducing SOX9 and blocking hypertrophy [118]. It is thought that non-canonical BMP signalling, via MAPK additionally contributes to matrix anabolism and



(caption on next page)

**Fig. 3. Schematic of developmental bone and cartilage formation.** (A) **Intramembranous ossification.** Aggregating mesenchymal cells differentiate into osteoblasts that establish an ossification center. As secreted osteoid calcifies, osteoblasts differentiate into osteocytes, which deposit hydroxyapatite and calcium to enhance mineralisation. Vascular invasion of the bone template results in spongy bone formation. Compact bone matures surrounding the spongy bone, maintained by osteoblasts. The surrounding mesenchyme forms the periosteum. (B) **Endochondral ossification.** BMP promotes mesenchymal aggregation and chondrogenic differentiation. Cells synthesise a collagen II/aggreccan-rich hyaline cartilage matrix and within the center of the cartilage anlage upregulate hypertrophic markers RUNX2 and type X collagen. VEGF induces vascularisation of the bone template. Invading blood vessels facilitate an influx of OSX1<sup>+</sup> osteoblasts and haematopoietic progenitor cells (HSCs). Hypertrophic chondrocytes induce hypertrophy in adjacent cells. Excavation and resorption of the cartilaginous matrix by HSC-derived osteoclasts generates the early bone marrow, determining the orientation of opposite distal epiphyseal growth plates. The growth plates are divided into zones with phenotypically distinct chondrocyte populations controlled by gradients of hypertrophic and inhibitory signals. (C) **Joint and articular cartilage formation.** The interzone cells, the site of joint morphogenesis, express GDF5 and WNT; inhibition of SOX9 leads to downregulation of *Col2a1* while a field of Noggin surrounding the interzone prevents chondrogenic differentiation. Joint cavitation occurs through synthesis of ECM, including hyaluronan, and migration of TGF $\beta$ R2<sup>+</sup> cells to the dorsal and ventral flanks. SOX9<sup>+</sup> cells from the anlage continue to transition into GDF5<sup>+</sup> cells and migrate to the interzone site.

cartilage stability through both gene induction and signalling crosstalk [119–121]. Non-canonical activation of p38 MAPK, through TGF $\beta$ -activated kinase (TAK1) and MAP Kinase Kinase 3/6 (MKK3/6), leads to phosphorylation of activating transcription factor 2 (ATF2), which binds to the *Sox9* promoter region, potentially as a co-factor with SMAD3 [120,122]. *Sox9* mRNA is stabilised by p38 MAPK, whilst in mice, *SOX9* protein activity is enhanced by interaction with P-SMAD1/5/8 [97,119]. Inhibition or conditional knockout of BMP pathway components *in vivo* leads to disruption of chondrogenesis and rapid degradation of articular cartilage [96,102,123,124].

#### 2.4. Molecular regulation of joint morphogenesis and articular chondrogenesis

Articular cartilage is generated and maintained by embedded articular chondrocytes that are phenotypically and functionally distinct from growth plate chondrocytes. Most notably, articular chondrocytes are arrested in a non-proliferative state and do not undergo hypertrophic maturation, enabling their retention within the cartilage. Throughout life, articular cartilage becomes degraded, as maintenance signalling networks become disrupted and the chondrocytes lose phenotype, resulting in a loss of joint function that often progresses to Osteoarthritis (OA) [125].

Articular chondrocytes, along with all other synovial joint tissues, arise from the interzone: a specific mesenchymal progenitor population that segments the embryonic anlage at the site of joint morphogenesis (Fig. 3C). The interzone consists of two chondrogenic outer layers perpendicular to the main limb axis and a mesenchymal-like intermediate zone [126]. Cells within the interzone downregulate chondrocyte-related genes, including *Sox9* and *Col2a1*, and present a distinct molecular phenotype including expression of *Wnt14/Wnt9a* and *Erg* [127]. Joint morphogenesis proper involves cavitation within the center of the interzone through synthesis of ECM such as hyaluronan, and expression of *Tgf $\beta$ r2* in dorsal and ventral flanking interzonal cells that precede the appearance of meniscal and other joint tissues [128, 129]. Continued signals from the interzone and surrounding anlage drive a phenotypic shift towards an articular chondrocyte fate, indicated through synthesis of structural proteins such as tenascin-C, type II collagen and receptor CD44, alongside expression of key markers including *Prg4* and *Bmpr1b* [130–132]. Emergence of lubricin (*Prg4*)-expressing articular chondrocyte progenitors, negative for Matrilin-1, signifies late-stage articular development and synthesis of a hyaline cartilage matrix. WNT and TGF $\beta$  signals drive expression of *Prg4*, both within articular progenitor and adult chondrocytes [133–135].

GDF5 is recognised as a key marker of joint morphogenesis and later, mature articular chondrocytes, but its role in *in vivo* chondrogenic development is poorly understood. GDF5 expression significantly diminishes after interzone formation, re-emerging upon articular chondrocyte maturation [136,137]. However, expression of the erythroblast transformation specific (ets) family member *ets-related gene* (*Erg/C-1-1*) is critical in driving an articular chondrocyte phenotype and acts downstream of GDF5 [138–140]. *Erg/C-1-1* is also critical in blocking

chondrocyte proliferation and hypertrophy, through downregulation of *Runx2* in combination with SOX9 [140,141].

GDF5 induces expression of chondrogenic genes, including *Sox9*, *Acan*, *Comp* and *Col2a1*, in addition to proteoglycan synthesis *in vitro* [142–144]. GDF5 can transduce signals through several BMP-like receptors, but has high affinity and binding preference for BMPR1B/ALK6 [145]. BMPR1B/ALK6 is expressed and plays an important role during mouse skeletal development, although its function overlaps and can be made redundant by BMPR1A/ALK3 [96]. However, the roles of BMPR1A/ALK3 and BMPR1B/ALK6 become distinguishable in adult cartilage, where both are primarily present within the superficial zone [131,146]. A recent study demonstrated that binding of a modified GDF5 with increased affinity for BMPR1B/ALK6 led to a strong downstream chondrogenic output with a high ratio of type II to type X collagen compared to signalling through BMPR1A/ALK3 [144]. The balance of signalling through BMPR1A/ALK3 or BMPR1B/ALK6 may strongly influence chondrocyte fate, with upregulation of BMPR1A/ALK3 associated with hypertrophy and onset of OA [144,147]. Other BMP family members have been demonstrated to exhibit pro-chondrogenic properties without induction of hypertrophy, such as BMP7 [148], and BMP9 in combination with BMP2 [149]. However, a BMP9-induced regenerative response required lubricin, indicating the importance of cellular context.

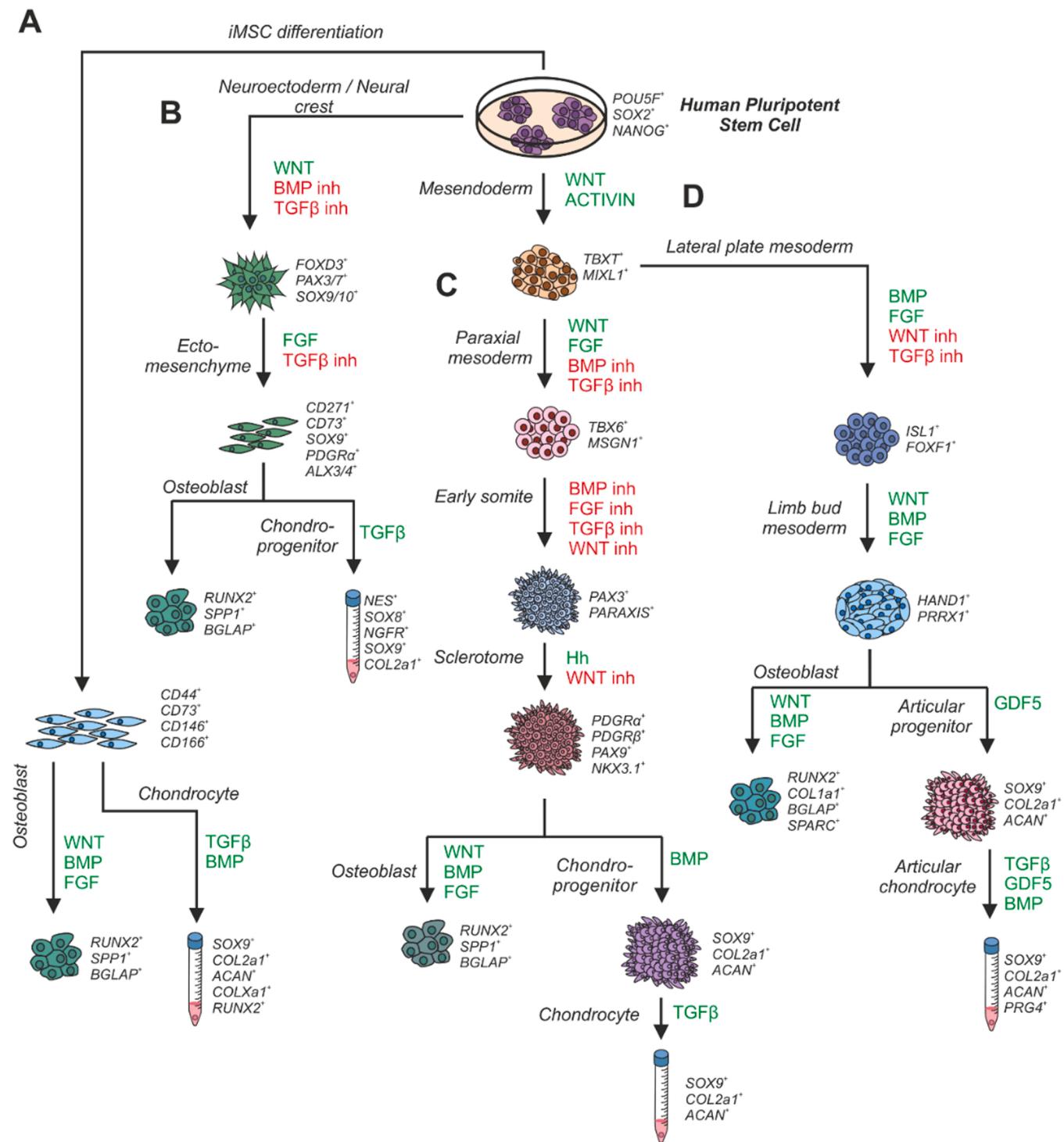
### 3. Generation of cartilage and bone *in vitro*

#### 3.1. Development of the field - early hPSC-differentiation to bone and cartilage

Many early attempts to generate cartilage and bone from hPSCs involved transition through an embryoid body (EB) stage, first established in murine ESCs. Induction of differentiation was performed with TGF $\beta$  family growth factors [150,151], or through co-culture with cells expected to produce factors promoting skeletal differentiation, such as chondrocytes [152]. However, early hESC-chondrogenic and -osteogenic differentiation attempts commonly used only partially defined media, focussed solely on growing cells in 2D and did not reflect the nuanced stepwise, location-specific differentiation and function-specific skeletal subtypes seen in developing mammals. Recapitulation of developmental mechanisms through stimulation with appropriate factors should generate cells that more closely resemble those developed in the embryo with a high capacity for matrix synthesis (Fig. 4). However, as growth factors are costly and suffer lability and batch-to-batch variation, most protocols now substitute these, where feasible, for small molecule pathway activators or repressors.

#### 3.2. Translation of developmental principles to human pluripotent stem cell differentiation

HPSGs can theoretically differentiate into any cell type through induction of developmental signalling pathways, and their application has led to the generation of increasingly efficient and reproducible differentiation protocols [153–155]. Although chondrocytes and osteoblasts



**Fig. 4. Overview of chondrogenic and osteogenic differentiation pathways of hPSCs.** A simplified schematic outlining differentiation of chondrocytes and osteoblasts through an (A) iMSC intermediate, (B) neural crest, (C) paraxial mesoderm and (D) lateral plate mesoderm. Activation (green) or inhibition (red) of signalling pathways indicated to drive hPSC differentiation towards each stage is highlighted. Examples of markers important to identify cell populations representative of each differentiation stage are shown (for details see [3]). (A) Mesenchymal-like cells (iMSCs) can be derived from hPSCs through adherent culture and then subjected to osteogenic and chondrogenic differentiation informed by development. (B) hPSCs can be directed through the neuroectoderm and neural crest route. Chondroprogenitors can be derived from ectomesenchymal cells. Similarly, osteogenesis can be induced with similar conditions to those utilised in adult MSC protocols. (C–D) Chondrocytes are more typically derived from primitive streak/mesendoderm-like cells; either paraxial (C) or lateral plate (D). (C) Paraxial-mesoderm generates somites from which the sclerotome is induced. In sclerotome BMP and TGFβ then induce chondrogenesis, whilst WNT, BMP and FGF drive osteogenesis. (D) Articular chondrocytes arise from limb mesenchyme during joint formation. The key interzone marker GDF5 can be used to induce differentiation towards articular chondrocyte progenitors. (C) and (D) Chondrocytes mature in pellet culture with appropriate growth factor stimulation. Limb mesenchyme-like cells can also be driven towards osteogenic differentiation through stimulation with WNT, BMP and FGF. Adapted from [2,154,164,170,173].

**Table 1**

List of protocols for the differentiation of hPSCs into cartilage or bone precursors, differentiation route, cell source, length of the differentiation and media compositions are reported. BDNF: brain-derived neurotrophic factor; EB: Embryoid body; FBS: Fetal Bovine Serum; SF: Serum-free; PS: Primitive streak

Author	Ref	Route	End product	Source	#Days	FBS	Stage	Medium composition
Lee 2007	[157]	Neural Crest	Cartilage/ Bone	hESC	70/ 102	10%	<b>Stage 1:</b> Neural induction <b>Stage 2:</b> Neural Crest Precursor <b>Stage 3:</b> Mesenchymal induction <b>Stage 4:</b> Chondrogenesis <b>Stage 5:</b> Osteogenesis	SHH, FGF8/FGF2, BDNF FGF2, BDNF none
Koay 2007	[150]	Unspecific	Cartilage	hESC	28	1%	<b>Stage 1:</b> EB <b>Stage 2a:</b> Chondrogenesis w/o COL1 <b>Stage 2b:</b> Fibrocartilage	TGFβ3 Osteogenic medium TGFβ3 TGFβ1, IGF-I
Nakagawa 2009	[208]	MSC	Cartilage	hESC	35	10%	<b>Stage 1:</b> Mesenchymal induction <b>Stage 2:</b> Chondrogenesis	TGFβ3 then BMP-2 FGF2
Evseenko 2010	[209]	Mesoderm	Cartilage/ Bone	hESC	38.5	SF	<b>Stage 1:</b> Mesoderm induction <b>Stage 2:</b> Mesenchymal Induction <b>Stage 3a:</b> Osteogenesis <b>Stage 3b:</b> Chondrogenesis	TGFβ1 and/or BMP-7 VEGF, bFGF, BMP4, Activin A, (FACS CD326-CD56 +) none (FACS CD73 +CD105 +CD34 –)
Oldershaw 2010	[154]	Mesoderm	Cartilage	hESC	14	SF	<b>Stage 1:</b> PS mesendoderm <b>Stage 2:</b> Mesoderm <b>Stage 3:</b> Chondrogenesis	WNT3A, Activin A, FGF2, BMP4 FGF2, BMP4, Follistatin, NT4
Umeda 2012	[174]	Paraxial mesoderm	Cartilage	hESC	32	1%	<b>Stage 1:</b> EB - mesoderm <b>Stage 2:</b> Chondrogenesis	BIO, Activin A or SB431542
De Peppo 2013	[210]	MSC	Cartilage/ Bone	hiPSC	35	20%	<b>Stage 1:</b> Mesenchymal Induction <b>Stage 2a:</b> Chondrogenesis <b>Stage 2b:</b> Osteogenesis	PDGF, TGFβ3, BMP4 none
Guzzo 2013	[211]	MSC	Cartilage/ Bone	hiPSC	21	10%	<b>Stage 1:</b> Mesenchymal Induction <b>Stage 2a:</b> Chondrogenesis <b>Stage 2b:</b> Osteogenesis	TGFβ3 Traditional osteogenic medium FGF
Craft 2013	[212]	Paraxial mesoderm	Cartilage	hESC hiPSC	26	SF/ 2%	<b>Stage 1:</b> EB formation <b>Stage 2:</b> PS-like mesoderm <b>Stage 3:</b> Paraxial mesoderm induction <b>Stage 4:</b> Chondroprogenitors <b>Stage 5:</b> Chondrogenesis	BMP2 Osteogenic medium BMP4 (hESC) / bFGF, Y-27632 (hiPSC) FGF2, Activin A, BMP4, CHIR99021 SB431542, bFGF
Koyama 2013	[213]	MSC	Cartilage	hiPSC hESC	42	10%	<b>Stage 1:</b> EB <b>Stage 2:</b> Monolayer culture <b>Stage 3:</b> Chondrogenesis	TGFβ3, BMP4 (From day 10) none none
Yamashita 2015	[184]	Mesendoderm	Cartilage	hiPSC	42	1%	<b>Stage 1:</b> Mesendoderm <b>Stage 2:</b> Chondrogenesis	WNT3A, Activin A
Loh 2016	[170]	Paraxial mesoderm	Cartilage/ Bone	hESC hiPSC	12	SF	<b>Stage 1:</b> Anterior PS <b>Stage 2:</b> Paraxial Mesoderm <b>Stage 3:</b> Early somite <b>Stage 4:</b> Sclerotome <b>Stage 5a:</b> Chondrogenesis <b>Stage 5b:</b> Osteogenesis	BMP2, TGFβ1, GDF5 Activin A, CHIR99021, FGF2, PIK90 A-83-01, CHIR99021, LDN-193189 [DM3189], FGF2 A-83-01, LDN-193189, C59, PD0325901 21 K, C59 BMP4
Loh 2016	[170]	Lateral plate mesoderm	Limb bud	hESC hiPSC	3	SF	<b>Stage 1:</b> Mid PS <b>Stage 2:</b> Lateral mesoderm <b>Stage 3:</b> Limb bud mesoderm	In vivo implantation Activin A, BMP4, CHIR99021, FGF2, PIK90 A-83-01 / SB-505124, BMP4, C59 BMP4, SB505124, CHIR
Jeon 2016	[214]	MSC	Cartilage	hiPSC	51	20%	<b>Stage 1:</b> EB formation <b>Stage 2:</b> Mesenchymal Induction <b>Stage 3a:</b> Chondrogenesis <b>Stage 3b:</b> Osteogenesis	none FGF
Kawai 2019	[200]	Unspecific	Bone	hiPSC	10	20%	<b>Stage 1:</b> Osteogenic Induction <b>Stage 2:</b> Osteogenesis	TGFβ1 Osteogenic medium Y-27632, RA
Wang 2019	[182]	Mesoderm	Cartilage	hESC	13	SF	<b>Stage 1:</b> Mesendoderm <b>Stage 2:</b> Mesoderm <b>Stage 3:</b> Chondrogenesis	RA WNT3A, Activin A, FGF2, BMP4 or BMP2 FGF2, BMP4 or BMP2, Follistatin FGF2, BMP4 or BMP2, GDF5
Lach 2019	[215]	Unspecific	Cartilage	hESC	40	20%	<b>Stage 1:</b> EB	FGF
Aisenbrey 2019	[216]	MSC	Cartilage	hiPSC	21	10%	<b>Stage 2:</b> Chondrogenesis <b>Stage 1:</b> Mesenchymal Induction <b>Stage 2:</b> Chondrogenesis	TGFβ3 FGF
Adkar 2019	[173]	Paraxial Mesoderm	Cartilage	hiPSC	49	1%	<b>Stage 1:</b> Anterior PS <b>Stage 2:</b> Paraxial Mesoderm <b>Stage 3:</b> Early somite <b>Stage 4:</b> Sclerotome	none / BMP2 and/or TGFβ3 Activin A, CHIR99021, FGF SB-505124, CHIR99021, FGF, DM SB-505124, DM, C59, PD173074 pumorphamine, C59

(continued on next page)

**Table 1 (continued)**

Author	Ref	Route	End product	Source	#Days	FBS	Stage	Medium composition
Diederichs 2019	[217]	MSC	Cartilage	hiPSC	70	12.5%	Stage 5: Chondroprogenitors Stage 6: Chondrogenesis Stage 1: Mesenchymal Induction Stage 2: Chondrogenesis Stage 1: Mesendoderm Stage 2: Chondrogenesis	BMP4 TGFβ3 bFGF TGFβ1 CHIR, TTNPB TTNPB
Kawata 2019	[187]	Mesendoderm	Cartilage	hiPSC	9	SF	Stage 1: EB formation Stage 2: MSC induction Stage 3a: Chondrogenesis Stage 3b: Osteogenesis	none none TGFβ3 Osteogenic medium
Xu 2019	[218]	MSC	Cartilage/Bone	hiPSC	43	20%	Stage 1: PS Stage 2a: Paraxial mesoderm Stage 2b: Lateral plate mesoderm Stage 2c: Neural Crest Stage 3: Osteogenic Induction Stage 4: Osteogenesis	CHIR99021 SB431542, LDN193189 BMP4, VEGF SB431542, CHIR99021 BMP2, FGF9, Rapamycin, WNT3a Traditional Osteogenic medium
Kidwai 2020	[201]	Paraxial mesoderm Lateral plate mesoderm Neural Crest	Bone	hiPSC hESC	28	SF	Stage 1: EB outgrowth Stage 2a: Chondrogenesis Stage 3b: Osteogenesis	none Minicircle for BMP2 and TGFβ3 Osteogenic medium
Rim 2020	[219]	Unspecific	Cartilage Bone	hiPSC	31	20%		

are often considered to be generic cell types, cells of distinct adult skeletal tissues can arise from several embryonic lineages (as discussed in [Section 2](#)). In order to ensure that hPSCs develop into a particular desired cell type, it has been important to understand chondrocyte and osteoblast lineages, what the necessary signals are *in vivo*, and how to translate this towards recapitulating the key steps of development *in vitro*. Ultimate validation requires transplantation *in vivo* and generation of authentic tissue of established implant origin.

### 3.2.1. Neural crest-derived craniofacial chondrocytes from hPSCs

NCCs generate most cranial skeletal tissue, and so could provide cartilage and bone for therapeutic use in craniofacial repair. NCCs were initially generated from hPSCs through co-culture before being further differentiated towards NCC derivatives e.g. sensory neurons [156], and mesenchymal progenitor cells (NCC-MPC) [157,158]. Alternatively, they can be derived from outgrowing EB cells, using NCC-induction medium containing epidermal growth factor (EGF), FGF2 and heparin [159]. NCCs can now be derived in defined conditions, giving more reproducibility, through BMP and TGFβ inhibition with Noggin and SB431542 respectively ([Table 1](#)) [158,160]. Small molecule activation of canonical WNT signalling, using BIO [160] or more specifically GSK3β inhibitor CHIR99021 [161], is critical in NCC specification. When WNT signalling is active, BMP inhibition has been reported as dispensable if applied in conjunction with TGFβ inhibition [161,162], using high cell density, or by controlling the endogenous BMP concentration [163].

Umeda and colleagues described a simplified method to derive chondrogenic cranial NCCs utilising cell sorting [164]. HPSC-derived SOX9<sup>+</sup>CD271<sup>+</sup>PDGFRa<sup>+</sup>CD73<sup>+</sup> ‘ectomesenchymal cells’ were isolated from a PAX3<sup>+</sup>/SOX10<sup>+</sup> NCC intermediate population, generated through stimulation with FGF and TGFβ inhibition. As well as chondrogenic markers (e.g. SOX9 and COL2A1) these cells expressed cranial mesenchyme markers, including ALX homeobox genes (ALX1/3/4), which are required for maintenance of the NCC-derived mesenchyme [165], and nerve growth factor receptor (NGFR), thought to be crucial for neuronal survival [166]. Upon stimulation with TGFβ, ectomesenchymal cells matured into hypertrophic chondrocytes expressing RUNX2 and COL10A1 *in vitro*, which fully mineralised once implanted into immunocompromised mice. A more recent simpler protocol generated NCC-MPCs through initial WNT stimulation and TGFβ inhibition, before culture in serum-containing (hence undefined) growth medium [167]. The NCC-MPCs exhibited chondrogenic, osteogenic and (to a lesser extent) adipogenic potential, as well as expressing

NCC-associated genes including *ALX3/4* and nuclear receptor subfamily 2 group F member 1/2 (*NR2F1/2*), known to be critical for NCC development [168]. Substantial chondrogenic and osteogenic characterization is required in the future, but these protocols hold promise for craniofacial repair interventions (see also [Section 3.2.5](#)).

### 3.2.2. Induction of a primitive streak-like stage

The majority of skeletal tissues arise from PS derived mesodermal lineages, [54,55]. Chondrogenic differentiation of hPSCs through both paraxial and lateral plate mesoderm therefore requires initial progression through a PS-like phase ([Sections 2.2.2 and 2.2.3](#)). Stimulation of TGFβ/Nodal receptor signalling with Activin A generates anterior PS-derived endoderm [169], whereas inhibition of TGFβ signalling suppresses its formation and promotes a hPSC mesodermal phenotype [170]. BMP and FGF stimulation of hPSCs result in posterior PS cell progeny, such as mesodermal tissues mediated by Brachyury and CDX2 [58,171]. Alternatively, hPSC WNT stimulation, through GSK3β inhibition, alongside FGF induces late PS and presomitic mesoderm markers whilst inhibiting formation of anterior cell types such as cardiomyocytes [172]. The application of increasing concentrations of BMP4 appears to promote a posterior PS-like phenotype and greater potential for lateral-plate-like mesoderm compared to hPSC derivatives not exposed to BMP which form anterior PS-like cells prone to generation of paraxial mesoderm [170].

### 3.2.3. Sclerotome-directed differentiation of bone and cartilage

Various research groups have reported robust differentiation protocols that direct hPSCs through paraxial lineages towards sclerotome-derived chondrocytes [173–176], summarised in [Table 1](#). One unresolved issue is the disparate cell surface markers (extrapolated from the developmental literature or human chondrocytes/progenitors) used in different studies to isolate enriched target populations, making protocol comparison challenging. Moreover, not all protocols have tested generation of appropriate fully characterized hard tissue *in vivo*. Early on, Umeda and colleagues demonstrated somitic/paraxial-chondrocyte differentiation through activation of WNT and TGFβ/Activin signalling, followed by SMAD inhibition and continued WNT activity [174]. A population of kinase insert domain receptor 2 (KDR2)<sup>-</sup> platelet derived growth factor receptor a1 (PDGFRa1)<sup>+</sup> cells were isolated and when subjected to PDGF, TGFβ and BMP generated a hyaline-like cartilage matrix [174]. When cultured with triiodothyronine and β-glycerophosphate, chondrogenic cells, induced with the Umeda protocol, progressed instead towards a hypertrophic phenotype [176]. The

**Table 2**

Examples of PSC skeletal disease models. Gene in which mutation occurs, differentiation route of protocol used, major phenotype of mutant differentiation and if any aspects of the phenotype were corrected in vitro are reported. TMAO: trimethylamine N-oxide, CBZ: carbamazepine, VPA: valproic acid.

Author	Ref	Disease	Gene	Route	iPSC phenotype	Phenotype corrected?
Quarto 2012	[238]	Marfan syndrome (MFS)	FBN1 (Fibrillin 1)	Unspecific chondrogenesis osteogenesis Osteogenic differentiation	MFS iPSC and hESC have impaired osteogenesis and chondrogenesis insensitive to TGF $\beta$ FOP iPSCs displayed increased mineralisation	TGF $\beta$ Inhibition
Matsumoto 2013	[225]	Fibrodysplasia ossificans progressiva (FOP)	ACVR1 (BMP type I receptor)			–
Saitta 2014	[234]	Metatrophic dysplasia	TRPV4	Unspecific chondrogenesis	Dom mutation (1604 M) reduced COL2A1 /PG, aberrant BMP2 response	–
Yamashita 2014	[229]	Achondroplasia SD; Thanatophoric dysplasia type 1	FGFR3	Mesendoderm chondrogenesis	Suppression of chondrocyte proliferation, increased apoptosis, abnormal differentiation, GAG degradation	FGFR3 inactivation, Statin
Okada 2015	[233]	Type II collagenopathy	COL2A1	Mesendoderm chondrogenesis	Lower COL2A1 expression, increased apoptosis, distended endoplasmic reticulum (ER) retained collagen type II.	TMAO, a chemical chaperone, improved collagen type II secretion and reduced BiP and CHOP expression
Xu 2016	[239]	Familial osteochondritis dissecans (FOCD)	ACAN (Aggrecan)	Teratoma	Accumulation of aggrecan in ER and ER stress	–
Esseltine 2017	[240]	Autosomal dominant developmental disorder oculodentodigital dysplasia (ODDD)	GJA1 (Connexin43)	Neural Crest Chondrogenesis osteogenesis	Delayed osteoblast differentiation, altered shape of chondrogenic micromass cultures	–
Kawai 2019	[200]	Osteogenesis imperfecta (OI)	COL1A1	Osteogenic differentiation	OI iPSCs displayed decreased mineralisation	mTOR inhibitor
Ozaki 2020	[232]	Achondroplasia	FGFR3	Mesendoderm chondrogenesis	Very limited chondrogenesis of mutant iPSCs	FGFR inhibitor ASP5878 improves chondrogenesis
Pretemer 2021	[176]	Metaphyseal chondrodysplasia type Schmid (MCDS) and multiple epiphyseal dysplasia (MED)	COL10A1 and MATN3	Sclerotome chondrogenesis	Retention of collagen 10 and MATN3	Partially CBZ, TMAO, VPN
Dicks 2021	[235]	Brachyolmia and Metatropic dysplasia	TRPV4	Paraxial Mesoderm chondrogenesis	Altered response to TRPV4 agonist GSK1016790A. Reduced hypertrophic maturation in response to BMP4.	–

authors have used the protocol to model chondrodysplasias with patient-derived iPSCs (discussed further in [Section 4](#); [Table 2](#)). Craft and colleagues used an EB intermediate to generate paraxial mesoderm, initially stimulating with Activin, BMP4 and FGF2 before BMP inhibition combined with FGF2 [175]. Cells expressing the key sclerotome markers *PAX1* and *NKX3.2* were induced with TGF $\beta$ 3 in micromass culture to form chondroprogenitors with some articular chondrocyte properties while BMP4 stimulation induced a number of growth plate markers [175].

Wu and colleagues identified an early articular chondrocyte progenitor population, with surface markers CD166<sup>low/neg</sup>/CD146<sup>low/neg</sup>/CD73<sup>+</sup>/CD44<sup>low</sup>/BMPR1B<sup>+</sup>, through microarray analysis of embryonic mouse limb [131]. Starting with a WNT-stimulated and BMP-inhibited hPSC -paraxial mesoderm population, enrichment of CD166<sup>low/neg</sup>/BMPR1B<sup>+</sup> cells, gave enhanced articular chondrocyte-like gene expression upon stimulation with leukaemia inhibitory factor (LIF) and TGF $\beta$ 1 [131]. More recently, the group utilised comparative RNA-seq and CHIPseq of 5 distinct fetal cell types including myoblasts, tenocytes, osteoblasts and 4 stages of chondrocytes *in vivo* with hPSC-early and -late (d60) chondrocytes *in vitro* [177]. Interestingly, the latter were staged as between fetal and adult chondrocytes. Presence or absence of integrin alpha 4 (ITGA4) was identified as a marker of unique populations within BMPR1B<sup>+</sup> chondrogenic populations. ITGA4<sup>-</sup>BMPR1B<sup>+</sup> cells exhibited the strongest matrix synthesis potential and were primarily localised to the transitional zone of articular cartilage. By contrast, ITGA4<sup>+</sup>BMPR1B<sup>+</sup> cells were robustly osteogenic, exhibiting osteochondral properties *in vitro*.

Most described protocols require several weeks to reach an immature

chondrogenic phenotype. Recently however, Loh and colleagues described rapid step-wise directed differentiation of hESCs towards several paraxial mesodermal derivatives (and lateral plate - [Section 3.2.4](#)) in days. They emphasised the need to block off-target, as well as stimulate on-target lineages [170], and pinpointed critical activating and repressing signals at different binary switch points for mesoderm differentiation, mapped using RNASeq, and ATACseq for chromatin status. Similar to previous work, WNT and FGF stimulation, with BMP inhibition, induced paraxial, and inhibited lateral plate mesoderm. Somite induction was executed through inhibition of WNT, BMP, TGF $\beta$  and FGF whilst exploiting single-cell RNAseq characterization for protocol optimisation, confirmation and efficiency. Sclerotome was specified through HH stimulation before chondrogenic induction with BMP, which mimics the sequence of signals required *in vivo* ([Section 2.2.2](#); [Fig. 2B](#)). Importantly, upon subcutaneous implantation into immunocompromised mice, hESC-derived sclerotome-like cells formed ectopic cartilage and bone. Xi and colleagues utilised a similarly rapid approach, forming hPSC somitic mesoderm in 4 days, by WNT pathway stimulation with CHIR99021, followed by BMP inhibition with LDN193189, and facilitated by SB431542 inhibition of SMAD2/3 – not predicted from model organism development [178]. Usefully, hPSC-somitogenesis was compared to RNAseq of human presomitic mesoderm and somites (4.5–5 weeks gestation). Sclerotome differentiation was induced with HH and FGF before *in vitro* chondro- or osteo-genesis. Osteoblasts expressed significant *RUNX2* and *COL1A1* and mineralized tissue stained for Alizarin Red, from day 20 of culture peaking at day 34. Chondrogenic pellets stained after 34 days for type II collagen and Alcian Blue for sulfated glycosaminoglycans (sGAGs) but no *in vivo*

analysis was reported.

Tissue specific reporters have enhanced efficiency in several protocols. For instance, a refined approach to further purify paraxial mesoderm-generated chondrocytes in vitro used a COL2A1-GFP knock-in reporter hiPSC line [173], enriching for COL2A1-GFP<sup>+</sup> cells after 9 days of BMP4 stimulation. After pellet culture for 28 days with TGF $\beta$ 3, a cartilage-like matrix rich in type II collagen and sGAGs was generated. Subsequently differentiating hPSC cultures were screened for CD146<sup>+</sup>/CD166<sup>+</sup>/PDGFR $\beta$ <sup>+</sup>/CD45<sup>-</sup> cells, extrapolated from native human chondroprogenitors. This enriched for a sub-population of highly chondrogenic cells with decreased heterogeneity [179]. More recently, single-cell RNAseq analysis of the Loh paraxial mesoderm protocol revealed some off-target differentiation, including neural cells and melanocytes within chondrogenic pellets. Selective inhibition of WNT signalling using C59, was reported to generate a more refined chondrogenic cell population [180].

Quantitative imaging-based optimisation of time/dose of CHIR99021 (GSK3 $\beta$  inhibition) and LDN193189 (SMAD1/5/8 inhibition), and controlling cell number, for hiPSC- paraxial mesoderm differentiation and somitogenesis [206] may well improve reproducibility and tissue quality as well as increase cost-effectiveness. The generated paraxial mesoderm aggregates from this approach closely resembled native tissue, developing into somite-like structures containing somitic cell types. However, formation of two parallel rows of somites produced in a unidirectional fashion has still to be achieved.

### 3.2.4. Generation of appendicular bone and cartilage from lateral plate mesoderm

Recapitulation of limb and joint developmental signals should lead to authentic articular chondrocyte-like cells, which arise from LPM. An early serum-free defined chondrogenic protocol devised by Oldershaw and colleagues drives hPSCs through mesendoderm and mesoderm stages with Activin A and WNT3a followed by BMP4 and FGF2, before stimulation with key interzone-associated growth factor GDF5 to generate articular chondroprogenitors [154]. Cells from this protocol can repair cartilage in osteochondral defects of RNU rats with good integration [181]. However, when grown in a 2D format, cells resembled immature chondroprogenitors expressing relatively low levels of COL2A1 and very low levels of type II collagen protein, much of which was released into the medium [182]. Substitution of BMP4 for BMP2 later in the protocol [182,183] improved COL2A1 expression, and in pellet culture with TGF $\beta$ 3, BMP2 and GDF5, modest synthesis of cartilage matrix proteins was achieved, including lubricin in surface cells as in articular cartilage [209]. Yamashita et al. [184] utilised suspension culture after initial chondrogenic differentiation of hiPSCs similar to [154], resulting in the formation of homogenous hyaline-like cartilage particles that generated cartilage blocks after implanting in joint defects of immunocompromised mice. Forcing specification of lateral plate and limb bud stages [185], as in the protocol used by Loh et al. [170], improved chondrocyte pellet formation, reportedly without prehypertrophic gene expression. Use of a PRRX1-reporter could be useful in selection of limb bud-osteochondral progenitors [92] (Section 2.2.3). Indeed, a recent study by Yamada and colleagues described a PRRX1-tdTomato reporter hPSC line, which enabled enrichment of expandable limb-bud-like mesenchymal cells with high chondrogenic potential [186].

Taking a different approach, a recent study demonstrated efficient differentiation of hPSCs to chondroprogenitors using only 2 small molecules: CHIR99021 and TTNPB, a retinoic acid receptor (RAR) panagonist (2C) [187]. CHIR99021 for 2 days initiated mesendoderm whilst continuous TTNPB stimulation resulted in chondrogenic differentiation, with significantly higher expression of SOX9, COL2A1 and COL11A2, compared to the protocols described above [154,184]. RAR signalling regulates epigenetic changes in hESCs through increasing histone acetylation levels, which likely facilitates differentiation [188]. However, the role of RA signals during chondrogenesis is poorly

understood, with conflicting reports of inductive or inhibitory effects [189–192]. No growth factor-targeted mesodermal specification was performed and a mixed paraxial/lateral plate mesodermal population was generated. These cells produced subcutaneous cartilage implants in vivo, but their ability to form cartilage in vitro was not reported. Therefore, the 2C protocol may be less suitable for developmental models, but may be promising for cartilage repair.

### 3.2.5. Osteogenic-differentiation

In vitro hPSC-osteogenesis protocols are primarily derived from those developed with bone marrow MSCs (BMSCs), where the standard procedure consists of using medium supplemented with dexamethasone (Dex), ascorbic acid (Vitamin C),  $\beta$ -glycerophosphate [193] and fetal bovine serum (FBS). Considering the length and complexity of human osteogenesis, it is unsurprising that cells require continuous stimulation for long periods in vitro. Three to four weeks of culture has been reported to improve both gene expression in vitro and in vivo osteogenesis [194]. Dex activates WNT/ $\beta$ -catenin signalling and induces RUNX2 expression [195], crucial for osteogenesis (Section 2.3.2) [196]. Ascorbic acid is an essential cofactor for the enzymes lysyl hydroxylase and prolyl hydroxylase, required for collagen synthesis, and, in stem cell-osteogenesis, is crucial for formation of the proper type I collagen organisation and secretion [193].  $\beta$ -glycerophosphate enhances mineralisation, acting as a phosphate reservoir for cells [193].

Given the more advanced differentiation state of BMSCs, generation of a progenitor would be prudent before use of these BMSC-osteogenic supplements with hPSCs. Although the importance of using lineage-specific cells for osteogenesis is recognised, differentiation of hPSCs with traditional osteogenic medium is still common. For instance, Zhou et al. [197] investigated alternative methods to analyse osteogenic differentiation of hPSCs using traditional osteogenic medium. However, the authors recognised that differentiation was poor due to the inadequacy of the induction, which generated a heterogeneous cell population. During endochondral ossification, hypertrophic chondrocytes direct matrix mineralisation through deposition of hydroxyapatite (HA), a prerequisite for invasion of blood vessels and osteogenic cells from the perichondrium. Consequently, the seeding of differentiating cells onto HA or tricalcium phosphate (TCP) substrates is another strategy used for osteogenic induction [198,199], but results have been disappointing compared to with BMSC osteoprogenitors. Kawai et al. [200] generated bone-like nodules in vitro using traditional medium supplemented with RA for a short period, which accelerated differentiation. After calvaria-grafting, cells differentiated without RA (but not with) generated fibrous teratoma-like tissue, indicating undifferentiated or partially differentiated cells. This highlights the fact that stimulation solely with osteogenic medium developed for BMSCs is insufficient for hPSC osteogenic differentiation.

More recently, Kidwai and colleagues [201] successfully differentiated osteoprogenitors through independent induction of the NCC, LPM and paraxial mesoderm. Cells were characterized with multiple lineage-markers and enriched by FACS to reduce heterogeneity. Initial induction of PS was performed using GSK3 $\beta$  inhibition (see Section 3.2.2). Based on the importance of differential BMP activity for mesoderm specification (Section 2.1.2; Section 2.1.3), BMP was applied (lateral plate) or inhibited (paraxial) for 6 days of lineage-specific induction with addition of VEGF, reported to facilitate hESC-lateral plate mesoderm [65,202]. Paraxial mesoderm was induced using SB431542-mediated TGF $\beta$  inhibition and LDN193189-mediated BMP/SMAD1/5/8 inhibition. NCC generation used WNT activation and TGF $\beta$  inhibition. Osteogenesis was then induced using traditional serum-free osteogenic medium but with WNT3A (for the first 6 days), BMP2, FGF9, and Rapamycin for 21 days. A RUNX2-YFP reporter facilitated evaluation (see below), and after 28 days of induction almost all cells were RUNX2 positive. Lineage-specific expression (e.g. of FGF7) was detected in LPM derived osteogenic cells. Importantly, differentiation was evaluated in vivo: following subcutaneous implantation into

mice for 16 weeks, osteoprogenitors derived from the different lineages showed different osteogenic fates. Neural crest-derived osteogenic cells, which expressed high levels of *FGF1*, did not appear to form chondrocytes, suggesting intramembranous ossification was occurring. In contrast, identification of chondrocytes from lateral plate and paraxial mesoderm differentiation 8 weeks after transplant suggested an endochondral route to ossification.

A major limitation in developing reliable methods for generating osteoblasts from hPSCs is the lack of unambiguous molecular markers for osteoblastic differentiation. This is due to the overlap of osteogenic cell surface markers such as CD105<sup>+</sup> [203], CD75<sup>+</sup> [204], CD90<sup>+</sup> [203] and BMPR1B<sup>+</sup> [177] with those for chondrogenic and multipotent cell lineages. In order to improve the efficiency of cell selection RUNX2-reporter lines are increasingly being utilised to identify osteogenic cells [205–207]. Using a RUNX2-YFP reporter line, a novel signalling axis was established for hPSC-osteogenesis through fibrinogen binding to integrin  $\alpha 9\beta 1$  (which facilitates bone marrow osteogenesis).  $\alpha 9\beta 1$ , expressed by RUNX2-YFP positive cells [206], activated SMAD1/5/8 phosphorylation and *RUNX2* expression. RUNX2 regulates expression of type I collagen, *ALP*, osteocalcin, osterix and *DLX5* [206]. Therefore, identification of RUNX2<sup>+</sup> cells using flow cytometry can be used to track hPSC osteogenic differentiation or optimise differentiation protocols through FACS-cell enrichment. These studies lay the foundation for development of efficient osteogenic strategies through different lineage routes.

#### 4. *In vitro* modelling of developmental genetic diseases of the skeleton

Genetic mutations affecting genes involved in skeletal development and cartilage ECM assembly can contribute to skeletal diseases ranging from rare monogenic dysplasia to common polygenic diseases such as OA. There are more than 300 skeletal dysplasias, which are commonly caused by a single rare mutation within the protein coding region of genes. By contrast, OA is the most common musculoskeletal disease, with more than 80 genetic loci known to contribute to OA disease phenotype [220]. Additionally, over 3000 genetic loci are associated with adult height, many of which map near genes with roles in skeletal development [221]. The causal small nucleotide polymorphisms (SNPs) are often within promoters of genes important in skeletal development, such as GDF5 or other genes involved in TGF $\beta$  signalling, and thought to alter transcription factor binding [222]. However, the exact mechanism by which many of these SNPs function is still unknown. Much of what is known about rare skeletal diseases is derived from mouse models [223]. Recent advances in iPSC reprogramming and differentiation of hPSCs to chondrocytes represent an alternative for the study of human skeletal diseases. However, current hPSC differentiation protocols almost invariably produce fetal-like tissue, so they are most valuable for understanding genetic diseases with early developmental phenotypes. HPSC models are particularly useful for the skeletal field due to difficulty in obtaining human tissue samples.

##### 4.1. Examples of PSC skeletal disease models

There are now several models of skeletal disease that utilise the differentiation protocols described previously (Section 3) to compare hiPSCs carrying skeletal genetic mutations with hiPSCs derived from unaffected individuals (Table 2). One of the first hiPSC models of a skeletal disease was for fibrodysplasia ossificans progressiva (FOP), a rare condition caused by hyperactive mutations within the BMP type-1 receptor ACVR1/ALK2, that leads to progressive ossification of soft tissues including skeletal muscle, tendon and ligaments [224]. By using iPSCs derived from patients with ACVR1/ALK2 mutations, Matsumoto and colleagues demonstrated that FOP iPSCs displayed increased mineralisation *in vitro* compared to non-mutant cells [225]. BAC-based homologous recombination to correct the ACVR1 mutation increased

BMP signalling in FOP-derived iMSCs. This was confirmed through determination of increased levels of pSMAD1/5/8 and luminescence from a transfected BRE-luc construct [226]. This research also lead to the discovery that Activin A can signal through ACVR1/ALK2 [227]. iPSC-models of FOP have been reviewed previously [228].

Yamashita and colleagues utilised iPSCs to model thanatophoric dysplasia (TD) type I and achondroplasia, caused by gain-of-function mutations of FGFR3. Thanatophoric dysplasia is a severe, usually lethal dysplasia. Accordingly, TD-iPSCs exhibited poor chondrogenic potential: differentiated tissue stained poorly for Safranin O and expressed very low levels of cartilage-associated transcripts, consistent with the severe human phenotype [229]. After transplantation *in vivo*, smaller hypertrophic chondrocytes in the growth plate were observed, as seen in patients [230]. The authors used the model to explore statins as a potential treatment for FGFR3 mutations [229]. Similarly, the FOP iPSC model has been utilised to screen candidate drugs to correct the ACVR1/ALK2 mutation, testing in e.g. iMSCs with this mutation [231], while the FGFR inhibitor ASP5878 was found effective at improving FGFR3-mutant iPSC chondrogenesis [232].

In mouse models for several skeletal dysplasias, mutations within genes encoding cartilage-associated ECM often cause protein misfolding, leading to mutant protein retention within the chondrocytes [223]. This was replicated in human iPSC models for *COL2A1* [233], *COL10A1* and *MATN3* mutations, although the induced ER stress appeared to be mutation specific [176]. TRPV4 ion channel mutations also cause skeletal dysplasia; TRPV4 mutant iPSC-chondrocytes respond differently during TGF $\beta$  and BMP induced chondrogenesis compared to unaffected controls, and to the TRPV4 agonist GSK1016790A [234,235]. This is consistent with the role of TRPV4 in BMP and TGF $\beta$  signalling [236, 237].

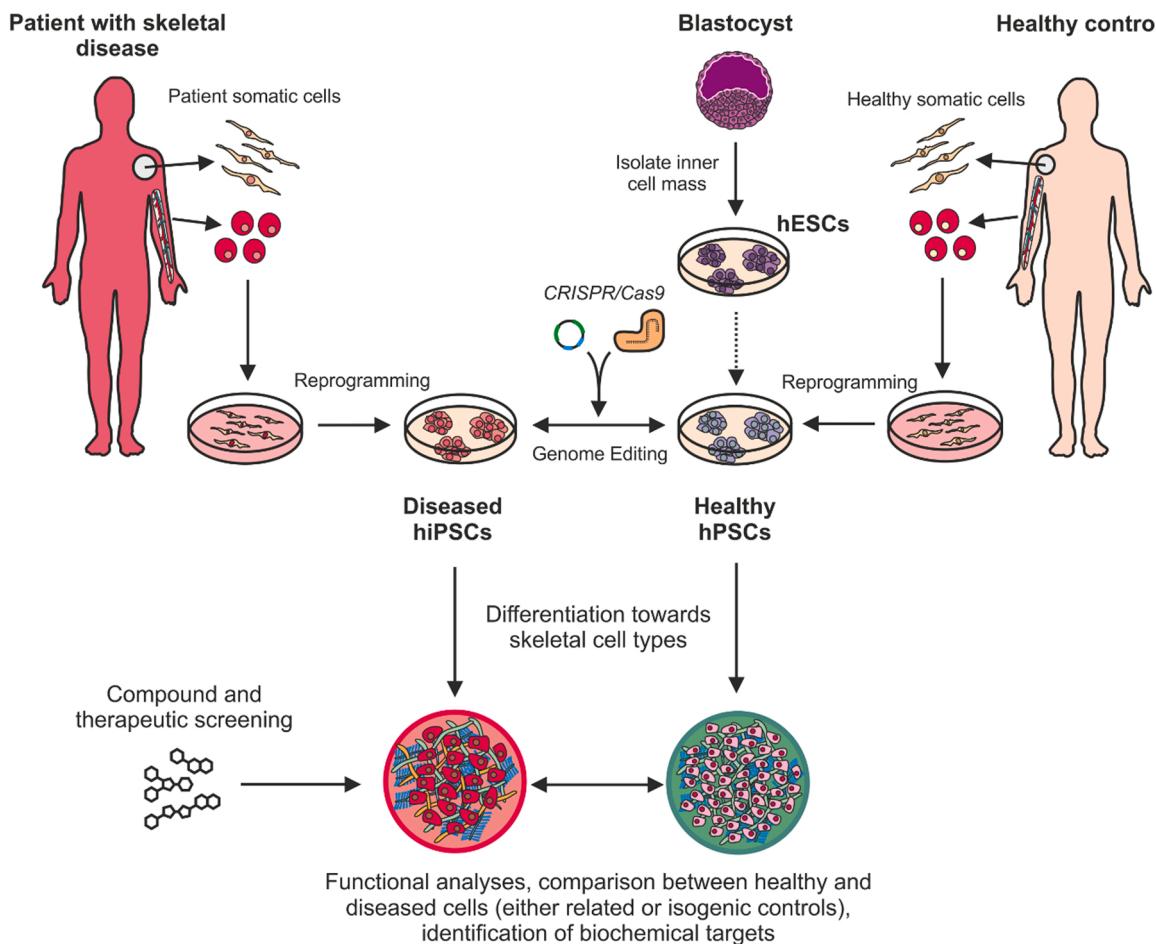
##### 4.2. Limitations and considerations of PSC skeletal disease models

###### 4.2.1. Developmental route and cell maturity

For the most part, current iPSC models of skeletal disease recapitulate the human phenotype remarkably well and have verified many of the findings from mouse models. However, key limitations remain. Cell phenotype will be affected by adjacent cells and even now, most protocols do not generate pure cell populations. Thus, off-target cells may facilitate or interfere with disease phenotypes. Many do not follow an authentic developmental route for differentiation to the affected tissue, which is likely to be an important consideration for the phenotype observed. For example, it is logical to model human conditions where the phenotype is restricted to the craniofacial skeleton by differentiation of cells through a neural crest-skeletal lineage. If the condition affects all chondrocyte types, the developmental route may be less important, although heterogeneous differentiation towards both permanent- and growth plate-like chondrocytes could still provide a source of variation between differentiation runs. All protocols are likely to produce chondrocytes at various stages of maturity/differentiation and thus, scRNA-seq is a promising technology to identify chondrocytes and osteoblasts most severely affected by a mutation. It is acknowledged that some epigenetic causes of disease may not be fully replicated in patient-hiPSCs, having been erased during reprogramming. However, this is not necessarily the case and phenotypic aberrations have already been replicated in differentiating mutant hiPSCs where epigenetic mediators are affected. For instance, hiPSCs with mutations in *CHD7* [241], a chromatin remodeller causing CHARGE syndrome, (30–50% of patients show skeletal anomalies), have already uncovered NCC aberrations. Rett syndrome patients with *MECP2* mutation have low bone mass and isogenic iPSC *MECP2*-mutant lines differentiate to neurons showing Rett pathology [242]. These and other models could be used in future for investigating bone and cartilage.

###### 4.2.2. Genetic background

Additional genetic mutations present due to genetic variance, may



**Fig. 5. Rationale of skeletal disease modelling with patient iPSCs.** Somatic cells are harvested from a patient with an identified skeletal mutation and reprogrammed into iPSCs that retain the disease-causing mutation. When differentiated towards skeletal cell types, the disease phenotype should manifest and enable functional analyses, identification of biochemical targets and high-throughput therapeutic screening. Diseased cells can be directly compared with iPSCs derived from an unaffected donor or hESCs from a blastocyst. For monogenic mutations, genome editing techniques can be used to correct or induce a mutation and establish isogenic cell lines, providing the most accurate phenotypic comparisons.

influence differentiation efficiency or modulate the effect of the primary causal mutation. To eliminate this effect, research groups have generated isogenic pairs of iPSC lines, either by correcting the mutation, or by making the mutation in healthy lines (Fig. 5). Although earlier studies used methods such as BACs [226], gene editing (e.g. CRISPR-Cas9) is currently the most efficient method; already used to generate iPSC lines carrying causal SD-mutations including in *COL1A1* [243], *COL2A1* [244,245] and *TRPV4* [235,246]. Well-controlled differentiation of isogenic lines is particularly important for studying skeletal diseases with mild phenotypes.

One of the immediate goals is to identify and screen drugs or compounds with potential to correct the mutant phenotype. For example, Hino et al. performed a high-throughput screen in ATDC5 cells, then tested candidates in an iPSC model [231]. Ozaki et al. reported that ASP5878, an FGFR inhibitor, promoted chondrogenesis of iPSCs harbouring a gain-of-function mutation in FGFR3 [231,232]. Enabling the use of an iPSC-skeletal model in a higher throughput format, rather than relying on transformed cell lines is desirable. The use of reporters in combination with mutant lines would be ideal for this, e.g. a SOX9 [247] or *COL2A1* [173,248] reporter. Indeed, *TRPV4*-mutant lines with a SOX9 reporter have been generated [246].

#### 4.2.3. Beyond monogenic mutations

Most of the current skeletal disease iPSC models are for rare monogenic conditions. It may however be possible to use iPSCs to model more

common polygenic conditions, such as OA and osteoporosis. Indeed, iPSCs have been derived from OA patients harbouring OA causal SNPs in *GDF5*, *SMAD3*, *ALDH1A2* and *IL1-R1* [249]. The OA patient-derived lines demonstrated limited capacity to produce ECM rich in collagen and proteoglycans. It is unlikely that a single OA-causal SNP will lead to detectable changes in iPSC-chondrogenesis using readouts of current models, but molecular changes, such as allelic imbalance in heterozygous iPSC lines when differentiated to chondrocytes should be detectable. Application of Chromosome-Conformation-Capture (3C)-based molecular techniques may reveal SNP function. Importantly, these findings can also be verified using CRISPR/Cas9, which is difficult in other models of human chondrogenesis e.g. adult MSCs. As well as inherited bone metabolic disorders [250], osteoporosis as an imbalance of bone mineral metabolism may also be tackled through iPSC approaches. For example, through generation of robust bone differentiation protocols, aimed at mechanistic insight, drug testing protocols or therapy. However, although there has been some progress, work is still needed to generate authentic iPSC-osteoclasts, so use of iPSCs to understand osteoporotic mechanisms awaits further research.

#### 5. Conclusions and perspectives

Developmental biology, over many decades, has extracted the principles by which tissues are laid down and organised in functional organs from model organisms. We have learned much of how vertebrate

skeletons are developed from chickens and mice and this has informed hypotheses for experiments on human cells, including hPSCs, aimed at understanding human development. Perhaps hPSCs form an ideal blank human slate here, whether set back to naïve, or primed and poised for differentiation (reviewed here [251]). They are particularly useful because of the ethical prohibition of experiments on human embryos beyond the earliest stages. Indeed, as we review above, extrapolation of key skeletal inducing factors from findings in non-human embryology and developmental genetics has successfully advanced our ability to generate hPSC-models of human skeletal cell development and disease. Protocols are becoming more efficient and reproducible. However, we still have to be vigilant for culture acquired mutations [252] as for other long term cultured cells. Moreover, whether the use of small molecules, or generic growth factors which are effective but developmentally inauthentic signalling activators, can always accurately replicate stage-specific human developmental signalling remains a question. For example, FGF2 has sometimes been the FGF family member used, irrespective of the developmental stage and range of active FGFs in vivo (Section 2.1.4); while small molecules invariably target one signalling pathway branch e.g. only the canonical WNT pathway. Are researchers using appropriate BMP(s) for making *human* neural crest or sclerotome and what are the roles of non-canonical growth factor signalling pathways in early hPSC-mesodermal tissue differentiation? Answers to these questions require further research. Development suggests a range of stage-specific skeletal progenitor markers but with species differences, making it hard to select appropriate sets for identification of human lineage-restricted articular cartilage or bone progenitors [3]: again a profitable study area. Interestingly, an expandable PRRX1 + population of hPSC limb bud-like cells for articular cartilage formation has recently been reported [186].

Still lacking is the technology to organise authentic morphogenesis in vitro, for example, parallel rows of somites, a directional growth plate, or an osteochondral joint containing different interacting cell types in the correct spatial arrangement. HPSC-bone constructs lack a marrow cavity, directional growth and necessary vasculature. There is still much work to do and scale may be important in recapitulation of proper structure and function; tissue engineering strategies or technological advances enabling dynamic, programmed signalling activation may aid us [3,253,254]. But we need to understand the variations in cell response to inducing factors, originating in the diverse human genomes and epigenomes of our hPSCs and humans themselves. This is a major factor in altered differentiation response between hPSC lines, together with in vitro history. Although current models are able to reveal aberrations in molecular pathways in monogenic skeletal disease; by using patient iPSCs or targeted CRISPR induced mutations, together with e.g., single-cell RNAseq, we are limited to models representing fetal-like bone and cartilage, despite development continuing post-natally. However, this is still a rich seam and combined with the rapid advancement in genetic approaches to understand chromatin regulation such as 3 C, ATAC-seq, single cell multiomics, cleavage under target and release by nuclease, chromatin immunoprecipitation and spatial transcriptomics will reveal much about healthy and aberrant human skeletal development.

Mice differ from humans in size (body mass ratio) relative limb length [255] and quadrupedal rather than upright bipedal locomotion, all pertinent to evaluation of the skeleton and its development. Body temperature and metabolism differ [255] including that of drugs [256] and their dose/kinetics or toxicity. This is a particular consideration in the use of other mammals or non-human cells in drug testing. So, well controlled reliable hPSC differentiation protocols, if taken to sufficient maturity and designed for high throughput application will be invaluable for biopharma.

Finally, much interest has focussed on use of hPSC derived cells for therapeutic use [257,258] and > 100 clinical trials are registered on the Clinical trials.gov website [259] under ‘pluripotent stem cells’ including for macular degeneration, Parkinson’s disease and heart failure. This

reflects the promise alluded to following the first reports of generation of hiPSC in 2007, 14 years ago. However, no trials appear registered for using hPSCs in skeletal applications. Though our differentiation protocols have improved greatly, there is a way to go before hPSC-therapies for traumatic or genetic skeletal conditions are economically feasible and sufficiently user-friendly and clinically efficacious for routine health care systems in mainstream medicine.

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