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Cartilage Regeneration Using Pluripotent Stem Cell-Derived Chondroprogenitors: Promise and Challenges

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Abstract

The cartilage of joints is long-lasting (i.e., permanent) cartilage and is not spontaneously repaired after injury in humans. There has been considerable interest in the clinical application of stem cells to the repair of damaged cartilage; however, current cell therapies using adult chondrocytes and mesenchymal stromal cells face problems associated with the low yield of such cells. The expansion culture, needed before transplantation, leads to the formation of fibrocartilage or growth plate-like (i.e., bone-forming) cartilage *in vivo*. Both types of cartilage are unsuitable for the repair of joint cartilage such as meniscus and articular cartilage. Joints are formed during embryogenesis. Therefore, we hypothesize that embryonic progenitor cells responsible for the development of joint cartilage would be the best for regenerating joint cartilage in the adult. Pluripotent stem cells (PSCs) are expected to differentiate in culture into any somatic cell types through processes that mimic embryogenesis, making human (h)PSCs a promising source of embryonic cells for regenerative medicine. However, regardless of the cell system used, the major research goals leading to clinical application to cartilage regeneration are to (1) expand chondrogenic cells (chondroprogenitors) to sufficient numbers without loss of their chondrogenic activity, and (2) direct the differentiation of such cells *in vivo* or *in vitro* toward articular or other types of chondrocytes of interest. The overall aim of the current review was to provide the basis of a strategy for meeting the goals for cartilage regeneration by the use of hPSC-derived chondroprogenitor cells. We provide an overview on signaling mechanisms that are known to affect the expandability and chondrogenic activity of adult and embryonic chondroprogenitors, as well as their differentiation *in vivo* or *in vitro* toward a particular type of chondrocyte. We then discuss alternative types of progenitor cells that might replace or combine with the hPSC-derived chondroprogenitors to regenerate permanent cartilage. We also include our recent achievement of successfully expanding hPSC-derived neural crest to generate ectomesenchymal chondroprogenitors that can be maintained for a long term in culture without loss of

chondrogenic activity. Finally, we provide information on the challenges that hPSC progeny-based regenerative medicine will face, and discuss the implications for such challenges for the future use of PSC progeny to regenerate cartilage.

Keywords: joint, cartilage, maturation, expansion, regeneration

1. Introduction

Damaged human joint articular cartilage does not heal spontaneously and eventually develops into osteoarthritis, probably due to a lack of proper "regenerative (i.e., stem/progenitor)" cells. Consequently, there has been considerable interest in the clinical application of chondrogenic stem/progenitor cells in the repair of damaged cartilage. For example, cell-based therapies have been developed that use the endogenous mobilization of marrow cells (i.e., microfracture method), or cells such as expanded (dedifferentiated) articular chondrocytes or mesenchymal stromal cells (MSCs) added exogenously via a periosteal patch or collagen membrane (i.e., autologous chondrocyte implantation method). Although such therapies relieve the major clinical symptom of pain, they do not promote the regeneration of long-lasting hyaline cartilage. Tissue engineering approaches that deliver a biodegradable matrix seeded with dedifferentiated chondrocytes or MSCs have been tested for the treatment of large cartilage lesions in animal models with similarly disappointing long-term results. Furthermore, the cells that persist at the repair site are often not the donor cells. Instead, they appear to be endogenous cells that have migrated from other sites in the body. The findings suggest that the transplanted chondrogenic cells may function as trophic mediators by stimulating endogenous repair functions. A trophic role would be problematic since there is no control for the selective migration of beneficial cell types such as articular chondrocytes and their precursors over unwanted cell types such as fibroblastic and osteogenic cells.

As to the cell type to be used for cartilage regeneration, chondrocytes are naturally suitable. However, their use requires *ex vivo* expansion since it is difficult to obtain sufficient chondrocytes for the treatment. During expansion culture, chondrocytes lose their characteristics. They dedifferentiate to fibroblastic cells that possess varying degrees of chondrogenic activity that quickly declines as culture continues. Furthermore, isolation of autologous chondrocytes requires an initial surgical removal of normal cartilage, which contributes to the increased risk of morbidity. MSCs isolated from adult bone marrow or fat tissue are defined *in vitro* according to both their differentiation capacity, typically to bone, cartilage and fat cell lineages, and their self-renewal activity [1, 2]. Adult muscle-derived stem cells (MDSCs) isolated from skeletal muscle can also be differentiated into bone and cartilage cells [3–5] in addition to other population of muscle progenitor cells [6]. Therefore, MSCs and MDSCs are expected to serve as precursors of chondrocytes (hereafter called "chondroprogenitors") and alternative sources of cells for cartilage repair. However, as in the case of chondrocytes, such chondroprogenitors also often require *ex vivo* expansion to achieve sufficient cells for the treatment. Furthermore, expansion of MSCs is known to result in losses in chondrogenic activity [7, 8] that can be decreased to some degrees by the inclusion of fibroblast growth factor (FGF) in the growth

media [9–11]. Another limitation is that the *ex vivo*-expanded adult MSCs tend to lose long-term viability and produce fibrocartilage tissue that is poorly integrated into the rest of the hyaline cartilage. Consequently, the repaired cartilaginous tissue is often lost from the site of damage, creating the need for subsequent surgery [12–15]. Attempts have been made to improve the *in vivo* chondrogenicity of adult MSCs and dedifferentiated articular chondrocytes by forcing the expression of genes that encode chondrogenic growth factors, for example, transforming growth factor (TGF)β and bone morphogenetic protein (BMP). While the attempts have led to short-term benefit, they have failed to prevent the developed chondrocytes from the hypertrophic differentiation, terminal maturation, and mineralization that promote osteophyte formation in the longer term [12]. So it seems that such *in vivo* factor-induced chondrocytes may be committed to form growth plate-like "unstable" cartilage [16]. Further attempts at improvement have aimed at controlling terminal maturation of chondrocytes and the ensuing osteogenesis process, such as by inhibiting angiogenesis using a soluble form of the vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1/Flt1) (while sFlt1 also increases the chondrogenic potential the cells) [3–5]. As stated by Somoza et al. [15], neither exogenously administered nor endogenously mobilized MSCs have contributed to the generation of durable and phenotypically correct (i.e., anatomic site-specific) articular cartilage. Evidence to date thus casts doubt on the potential usefulness of MSCs in the regeneration of hyaline articular cartilage. A better source of chondrocytes and better knowledge of the critical environmental cues for the development of articular cartilage by chondrocytes are clearly needed [17]. Regardless of the type of chondrogenic cells used, the main challenges with cell-based, articular cartilage tissue-engineering remain: (1) quality and durability of the *de novo* generated cartilage tissue, (2) resistance of the tissue to an endochondral ossification program, and (3) its effective integration into the anatomic site-specific host tissue.

Joint formation is initiated during embryogenesis by a specialized cell type, the "joint progenitor," which is responsible for generating hyaline articular cartilage. Furthermore, surface injury that is introduced *in utero* into fetal joint cartilage is repaired spontaneously without scarring in a large animal model [18] indicating that embryonic epiphyseal chondrocytes possess spontaneous repair activity. It is thus conceivable that embryonic joint progenitors and/or chondroprogenitors may elicit better regenerative capacity than adult dedifferentiated chondrocytes or MSCs at the injured cartilage even in the adult. However, the only practical source of such specialized embryonic cells from humans would be pluripotent stem cells (PSCs). Whether derived from an embryo (embryonic stem cells or ESCs) or induced from adult tissue cells (induced pluripotent stem cells or iPSCs), PSCs can be maintained in culture without loss of their pluripotency and are expected to differentiate in culture into any type of somatic cell. Interestingly, both types of PSC represent inner cell mass or epiblast so that their *in vitro* differentiation mimics early embryogenic processes [19–21]. Therefore, it is expected that human embryonic progenitors involved in joint cartilage formation can be generated using the wealth of knowledge accumulated from studies on the developmental biology of bone and cartilage. In general, PSCs are induced to differentiate into mesenchymal chondroprogenitors prior to chondrocyte formation. However, most of the early studies employed spontaneous (i.e., uncontrolled) differentiation methods to generate such mesen-

chymal cells, followed by expansion culture using serum-containing MSC media to enrich them [22]. Unfortunately, such expansion was accompanied by the loss of chondrogenic activity [23, 24], as seen with adult human chondrocytes and MSCs [25, 26]. Furthermore, some reports appear to have overlooked knowledge of embryology and treated PSCs as if they were limb mesenchyme or MSCs by trying to induce chondrogenesis directly in a TGFβ-containing chondrogenesis medium designed for inducing chondrogenesis from adult MSCs [27]. Consequently, potential benefits for cartilage repair by human (h)PSC-derived chondroprogenitors over those by adult hMSCs, whether in quantity or quality, have not been demonstrated [22]. We proposed that the disappointing early observations may have been attributable to the use of mesenchymal hPSC progeny of unclear embryonic origins ["Origin matters" 28], and undefined conditions for their expansion. The choice of both cell type and medium failed to draw on the wealth of available information on the signaling mechanisms involved in embryonic skeletogenesis, thereby hampering progress toward the effective repair of cartilage. Recently, reports including ours [23, 29–35] have been based on knowledge of embryology to generate chondroprogenitors of a particular embryonic origin and the use defined culture conditions for their expansion.

Concerning the challenges with the use of MSCs as stated by Somoza et al. [15], it is conceivable that the successful repair of articular cartilage will depend on both the ability of chondrogenic cells with high expansion capacity to form hyaline cartilage and a microenvironment at the site of repair that promotes chondrogenesis, while preventing terminal maturation and mineralization of chondrocytes. Therefore, we aim to give an overview on what we know from genetic as well as cellular (e.g., MSCs and MDSCs) studies, (1) about ways to expand chondroprogenitors and maintain their chondrogenic activity, and (2) about the potential signaling mechanisms and cell populations that affect the type (e.g., articular chondrocytes or growth plate chondrocytes) and/or differentiation state (e.g., immature or mature) of chondrocytes generated from chondroprogenitors. We will also summarize relevant information obtained from our studies of hPSC-derived chondroprogenitors.

2. Long-term expansion of chondroprogenitor cells without loss of their chondrogenic activity: advantages of hPSC-derived progenitors

With any cell system, one of the major research goals for cellular therapy or tissue-engineering approach for cartilage regeneration is to expand chondroprogenitors to yield large numbers without the loss of chondrogenic activity. These goals are important because the quality and durability of the *de novo* generated cartilage tissue seem to be inversely related to the extent of expansion culture of articular chondrocytes and MSCs. Retention of chondrogenic activity even after long-term expansion in adult stem/progenitor cells is not a trivial outcome. Standard serum-containing growth conditions support chondrogenic activity of MSCs for only 2–4 passages. However, attempts to circumvent the problems by improving the MSC culture conditions have met with some success. Here, we will provide an overview of the studies done with MSCs and hPSC-derived progenitor cells. As expected, under optimal

culture conditions, the proliferative potential of PSC-derived progeny far exceeds that of their adult counterparts.

2.1. FGF, TGFβ, hedgehog, and WNTs for the expansion of adult MSC

FGF signaling is known to upregulate expression of *Sox*9, a key regulator of chondrogenesis [36–38], in chondrogenic mesenchymal cell lines and primary chondrocytes [39]. FGFs are also known to preserve the ability of limb-bud mesenchymal cells to undergo chondrogenesis, when the WNT signal is removed [40]. On the other hand, FGF2 does not consistently affect *in vitro* chondrogenesis from MSCs [41, 42]. However, FGF2 supplementation during expansion culture in the presence of 10% (v/v) fetal bovine serum (FBS) reproducibly enhanced their growth [9–11] and maintained the chondrogenic activity up to 7 passages for hMSCs [11] and up to 9 for rabbit MSCs [9] as assessed by the standard TGFβ-induced *in vitro* chondrogenesis culture. Serum-free methods for the expansion culture have also been established. The StemPro-based expansion culture for hMSCs from adult bone marrow sustained their growth, morphology, and tri-lineage (bone, cartilage and fat) potential for at least 5 passages [43]. In addition to FGF2, the StemPro culture contains platelet-derived growth factor PDGF-BB, TGFβ1, and insulin. Therefore, inclusion of FGF2 in the growth medium for hMSCs will be beneficial for expanding hMSCs for cartilage repair.

Indian hedgehog (Ihh) is a key mitogen for chondrocytes in the growth plate [44]. Similarly, hedgehog signaling stimulates the growth of human bone marrow MSCs and enhances chondrogenesis in serum-containing culture conditions [45]. On the other hand, WNT signaling is known to play important roles on the growth, specification, movement, and organization of early precursor cells for osteochondrogenesis [46]. The roles of WNT signaling on the proliferation of embryonic osteochondrogenic mesenchymal cells [47] and their association with osteogenesis have been well established [48, 49]. Very recently, WNT3a has been shown to further stimulate the FGF2-stimulated growth of hMSCs and enhance the FGF2-enhanced maintenance of chondrogenic potency for at least 20 days (4 passages) of expansion culture in the presence of 10% FBS [50]. In spite of these positive results with hedgehogs and WNTs in expanding mesenchymal cells, both factors are "developmental factors" that induce osteogenesis rather than chondrogenesis during skeletogenesis. Through the canonical signaling pathway (involving β-catenin), WNTs reduce Sox9 levels and antagonize the functions of Sox9 in chondrocytes [51], and Ihh is placed upstream of the WNT/β-catenin signaling events, essential for osteoblastogenesis in the perichondrium of developing bone [52–55]. We have also found that WNTs stimulate the growth of hPSC-derived chondroprogenitor cells (data not shown). Although the effects may depend on the developmental/differentiation stage of the target cell, use of WNTs and Ihh on chondrogenic mesenchymal cells probably needs careful prior examination to verify that they do not cause loss of chondrogenic activity during expansion. Research into ways of improving expansion culture for hMSCs to preserve their chondrogenicity reproducibly is still ongoing.

2.2. Suppression of TGFβ signaling successfully expands endothelial (progenitor) cells derived from PSCs

Mesenchymal progeny of PSCs had the similar problems as adult MSC, namely the loss of original phenotypes and developmental potentials during expansion. In this regard, Miyazono and colleagues [56] published a pioneering work in 2003 in which they demonstrated that Nodal/Activin/TGFβ receptor kinase inhibitor, SB431542, enhanced the growth and integrity of mouse (m)ESC-derived endothelial cells, leading to a successful protocol for the expansion of hPSC-derived endothelial cells [57]. TGFβ is known to be a potent inhibitor for lymphohematopoietic progenitor cell proliferation [58]. However, as the name "transforming growth factor" indicates, it was originally found as a potent growth-promoting factor for many transformed (tumor/cancer) cells and untransformed (normal) cells, including human adult bone marrow MSCs [43]. PSC-derived endothelial cells may produce TGFβ-like activity that serves as a "brake" on their own proliferation in culture.

2.3. Long-term expansion of endoderm stem/progenitor cells derived from hPSCs without loss of their developmental potency

Recently, multipotential endodermal stem/progenitor cells, which can undergo long-term expansion without loss of their phenotypes and developmental potentials (i.e., can self-renew), have been developed from hPSCs [59, 60]. The foregut stem cells can be maintained in culture over 18 passages in RPMI + B27-based serum-free medium supplemented with FGF2, Activin A, BMP4, hepatocyte growth factor (HGF), and epidermal growth factor (EGF) [60]. The endodermal progenitor cells can be maintained over 24 passages in IMDM:F12 (3:1) + N2/B27-based serum-free medium containing FGF2, BMP4, VEGF, and EGF [59]. Nodal/Activin/TGFβ receptor kinase inhibitor is not necessary for the long-term expansion of endodermal stem/progenitor cells. Therefore, the inhibition of Nodal/Activin/TGFβ receptor kinase is not a common requirement for the expansion of hPSC-derived progeny.

2.4. Long-term maintenance of hPSC-derived chondrogenic ectomesenchymal cells without loss of their chondrogenic activity

In contrast, methods for the long-term expansion of hPSC-derived chondrogenic mesenchymal cells had not been explored extensively. Research has focused more on the "genesis" but not "expansion" of chondrogenic activity from hPSCs. Although a short-term expansion of chondrogenic activity as judged by the increase in the SOX9$^+$ cell number was reported [61], expansion of PSC-derived mesenchymal cells has not been accompanied by long-term maintenance of their chondrogenic activity in the conventional MSC medium, as in the case of adult MSCs [22]. Proliferation without loss of chondrogenic activity is thus not an intrinsic property of the hPSC-derived "embryonic" chondrogenic cells. Rather, it is a property that can emerge under certain defined culture conditions. In this respect, we have previously established and refined signaling requirements for the differentiation of mouse and human ESCs and iPSCs to chondrogenic lateral plate mesoderm, paraxial mesoderm, and cranial neural crest-like progeny in a serum-free defined medium [22, 31, 33–35, 62–64]. Our group was the first to define the MIXL1-green fluorescent protein (GFP)$^+$ VEGFR2 (KDR)$^-$ PDGF

receptor (PDGFR)α^+ human paraxial mesoderm progeny and T-GFP$^+$KDR$^-$PDGFRα^+ mouse paraxial mesoderm progeny, from which chondrogenic mesenchymal cells were derived [33–35, 62]. We have also recently reported that MIXL1-GFP$^-$CD271^{hi}CD73$^-$ human neural crest-like progeny develops in a chemically defined medium [CDM: IMDM:F12 (1:1), 0.5% (w/v) fatty acid-free BSA, 1% (v/v) synthetic lipids, 10 µg/ml insulin, 300 µg/ml holo-transferrin, 0.17 mM ascorbic acid-2-phosphate, 0.3 mM monothyoglycerol] in the presence of Nodal/ Activin/TGFβ receptor inhibitor (SB431542) [65–68] and that they quickly become CD271$^+$CD73$^+$ ectomesenchymal cells expressing *SOX9* (albeit at lower levels than those achieved during chondrogenesis) during expansion in CDM in the presence of FGF2 and SB431542 (FSB condition, **Figure 1**) [31]. Significantly, such cells maintain their normal karyotype and chondrogenic activity at least at passage 10 and passage 16, respectively. In contrast, removal of SB431542 slows down or stops the proliferation with enhanced expression of *TGFB1/2/3* genes and TGFβ target genes such as *TGFBI* and decreases their chondrogenic activity after 2–4 passages. Removal of FGF, the only growth factor in the medium, led to immediate cessation of growth. Thus, the neural crest-derived ectomesenchymal cells originating from hPSCs are similar to PSC-derived endothelial cells in that the autocrine or paracrine action of TGFβ serves as an intrinsic "brake" on their expansion in culture. In order to shed light on the molecular mechanism of such long-term growth and maintenance of
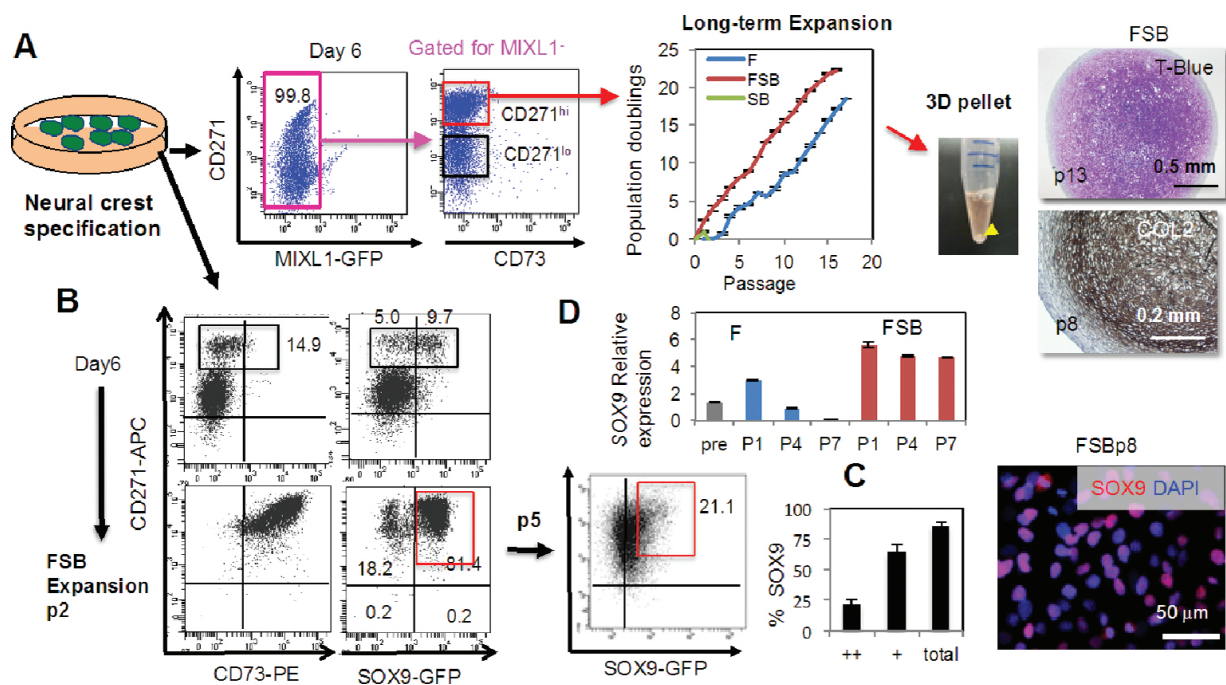
Figure 1. (A) Chondrogenic neural crest-ectomesenchymal cells. The MIXL1-GFP-CD271^{hi}CD73$^-$ cell fraction, enriched in the *SOX10* transcript, was isolated and expanded under FSB to generate SOX9-expressing chondroprogenitors. The formed cartilage particles were fixed, sectioned and stained with Toluidine blue (T-Blue) and anti-COL2 antibody. N: Noggin, SB: SB431542, F: FGF2, C: CHIR. (B) Ectomesenchymal cells from the SOX9-GFP hiPSC line. The iPSCs were differentiated into CD271^{hi}CD73$^-$ neural crest-like progeny: 2/3 of which (9.7%) are SOX9-GFP$^+$, were then expanded in FSB, leading to CD271$^+$CD73$^+$ cells that are mostly SOX9-GFP$^+$ (81.4%), but reduced to mostly SOX9-GFP^{lo} by passage (p) 5. (C) Immunostaining supported the FACS results. ++:high, +: low (D) *SOX9* mRNA expression was stably maintained by p7.

chondrogenic activity in culture, we performed genome-wide transcriptome analysis using RNA-seq technology to compare RNA profiles of ectomesenchymal cells cultured under FSB conditions with those of cells initially maintained in FSB but later exposed briefly to media without SB431542 (FSB → F) [31]. Bioinformatic analysis has revealed a list of protein-coding genes and non-coding RNA genes specifically expressed in cells cultured under either condition (**Figure 2**). Among them are growth-promoting genes, such as *LIN28A/B* and *MYCN*, neural crest/ectomesenchymal genes, such as *NGFR, TWIST1/2, ALX1/3/4,* and *SOX8,* and *SOX9* regulator genes, such as *MAF, ZBTB16,* and *LBH* [31].
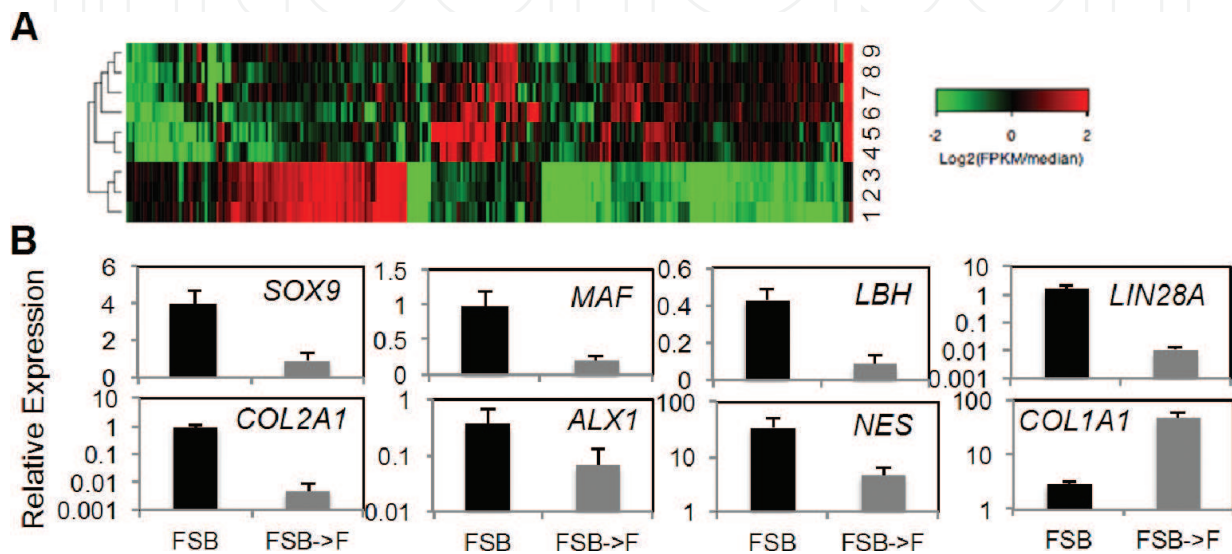
Figure 2. Comparative transcriptome analysis of ectomesenchymal cells using the RNA-seq technology. (A) Heat map of the top 250 genes, which are differentially expressed among the three groups. Lanes 1–3;"FSB", Lanes 4, 5, 8; "F", Lanes 6, 7, 9; "FSB→F". (B) RT-PCR confirmation of the relative gene expression levels predicted by the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values. F: FGF2, SB: SB431542, →: transition of culture conditions.

In contrast to PSC-derived endothelial cells and chondrogenic ectomesenchymal cells, the maintenance of human adult bone marrow MSCs in culture required TGFβ in a defined culture conditions [43]. Therefore, PSC-derived ectomesenchymal cells and bone marrow MSCs are distinct cell types, despite the proposal that both may share the common developmental origin of neural crest [69–71]. In support, application of SB431542 to mouse bone marrow MSC culture failed to support their growth and preferentially left adipocytes (data not shown), consistent with the notion that TGFβ suppresses adipocytic differentiation of mesenchymal progenitors [72].

3. Signaling mechanisms potentially leading to the genesis of long-lasting immature cartilage from hPSC-derived chondroprogenitor cells

The reproducible generation, either *in vivo* or *in vitro*, of articular chondrocytes that form immature, unmineralized cartilage that can be maintained in the long-term *in vivo* (designat-

ed hereafter, permanent cartilage) also remains a difficult task. For example, while articular chondrocytes, whether uncultured or briefly cultured, generate *in vitro* hyaline cartilage with no sign of hypertrophic differentiation and terminal maturation [73, 74], MSCs appear to be limited to an endochondral ossification program under the same condition—a program that will result in vascular penetration, marrow deposition, and ossification of the developed cartilaginous tissue [15, 49, 74, 75]. The critical environmental signals required for pathway switching toward the genesis of permanent hyaline cartilage have not been discovered. In this respect, it is worth noting that the differentiation state of chondrocytes generated *in vitro* from MSCs affects the stability of generated cartilage after transplantation. When MSCs are induced to generate mature, hypertrophic chondrocytes *in vitro*, ossification of the developed cartilage pellets *in vivo* is accelerated [16]. Therefore, controlling the hypertrophic differentiation of developed chondrocytes seems to be a promising first step toward the generation of permanent cartilage. Interestingly, even in the endochondral ossification process, there are signaling mechanisms that inhibit chondrocyte hypertrophic differentiation [49]. Such mechanisms have been defined via extensive genetic studies. The process of hypertrophic differentiation and terminal maturation is initiated by signaling events that eventually suppress chondrocyte determinants such as Sox9 and express determinants of the osteogenic program, such as Runx2 in chondrocytes. Sox9 is the master transcription factor for chondrocytes [36, 37, 76, 77]. Runx2/Cbfa1 is the master regulator of "mineralization" in both chondrocytes and osteoblasts [78–80] and facilitates hypertrophic differentiation of chondrocytes (e.g., stimulates the expression of *Col10a1* the marker for chondrocyte hypertrophic differentiation) in the growth plate [81–85]. Any of the signaling mechanisms that maintain Sox9 without inducing Runx2 or other osteogenic transcription factors in chondrocytes could be the pathway switcher for MSCs, which we believe should also help to generate permanent cartilage from hPSC-derived chondroprogenitors. We have summarized the roles of five well-known signaling mechanisms below that affect endochondral ossification *in vivo* and chondrogenesis from MSCs *in vitro* and the implications for each for chondrogenesis from hPSC-derived chondroprogenitors.

3.1. Indian hedgehiog (Ihh)-parathyroid hormone-related peptide (PTHrP) feedback loop

The Ihh-PTHrP feedback loop is one of the best-known mechanisms that controls chondrocyte hypertrophic differentiation in the growth plate [86–89]. After the cartilage primordium has formed during embryogenesis, perichondrial cells and chondrocytes at the ends of the cartilage produce PTHrP that signals through PTH receptors on chondrocytes. PTHrP action keeps chondrocytes in a proliferative state and delays their differentiation toward the postmitotic hypertrophic chondrocytes [90]. The chondrocytes distant from the cartilage ends escape from such PTHrP effects and stop proliferating (pre-hypertrophic chondrocytes). Such chondrocytes synthesize Ihh, which in turn stimulates the synthesis of PTHrP from chondrocytes at the ends of the cartilage, potentially through the action of TGFβ [91, 92]. The pre-hypertrophic chondrocytes differentiate to hypertrophic chondrocytes and then to terminally matured, mineralized chondrocytes. The feedback loop between PTHrP and Ihh thus regulates the pace of differentiation of immature (proliferating) chondrocytes. The Ihh-PTHrP system may also function similarly during articular chondrogenesis [93].

Elucidation of the individual roles of hedgehog and PTHrP signaling is important if there is to be future application of the Ihh and PTHrP signaling mechanisms to control chondrocyte hypertrophic differentiation during chondrogenesis from MSCs- or hPSC-derived chondroprogenitors. To date, the role of hedgehog signaling on chondrocyte maturation appears contradictory. Ihh directly stimulates proliferation of (immature) chondrocyte but also stimulates osteogenesis from osteochondrogenic mesenchymal cells. In addition, in the absence of PTHrP, Ihh appears to stimulate hypertrophic differentiation of chondrocytes through activating canonical WNT signaling (analogous to the Ihh-induced osteogenesis process) [94, 95] and vascularization of the hypertrophic cartilage, leading to trabecular bone formation [44, 96]. However, the overproduction of Sonic hedgehog (Shh) in cartilage seems to upregulate Sox9 directly in chondrocytes and chondrogenic mesenchymal cells and prevents or slows the maturation of chondrocytes [97, 98].

In contrast, the effect of PTHrP on the suppression of chondrocyte hypertrophic differentiation seems clear. The critical mechanism of PTHrP in keeping chondrocytes proliferative and delaying their hypertrophic differentiation is the activation of Gs and adenylyl cyclase to increase the level of intracellular cyclic adenosine monophosphate (cAMP) [99]. The rise in cAMP leads to the suppression of Kip2 cyclin inhibitor expression [100] and activation of Sox9 by cAMP-dependent protein kinase (PKA)-mediated phosphorylation [101, 102]. PTHrP decreases the level of *Runx2* mRNA [103] and stimulates the degradation of Runx2 protein [104]. The activated Sox9 [101, 102] antagonizes Runx2 function via Nkx3.2 protein [105, 106] or facilitates its degradation [107]. The delay in chondrocyte hypertrophic differentiation by PTHrP is not only dependent on the inhibition of Runx2 function and expression [108]. Histone deacetylase 4 (Hdac4), when phosphorylated by Ca^{++}/calmodulin-dependent protein kinase II (CaMKII), stays in the cytoplasm of chondrocytes, where it is dephosphorylated by protein phosphatase 2A that is activated by PTHrP signaling through the cAMP–PKA pathway. Dephosphorylation of Hdac4 facilitates its translocation to the nucleus, which leads to the inhibition of Mef2c action and suppression of chondrocyte hypertrophic differentiation (e.g., suppression of *Col10a1* expression) [109]. Interestingly, *Pthrp* mutant mice show mineralization of nasal cartilage [93]. Nasal cartilage is permanent cartilage. Therefore, one of the mechanisms by which permanent cartilage is maintained is via the activation of PTHrP signaling. Thus, the mechanism of PKA-mediated suppression of hypertrophic differentiation of chondrocytes is well established.

Investigation of the effects of PTHrP on *in vitro* chondrogenesis from MSCs, especially on the suppression of hypertrophic differentiation of developed chondrocytes, has, however, yielded contradictory results. On the one hand, PTHrP was found to enhance hyaline chondrogenesis by maintaining type II collagen (COL2) expression and suppressing the expression of type I (COL1) and type X collagens (COL10) [110]. On the other hand, PTHrP suppressed the gene expression of both *COL2A1* and *COL10A1* during *in vitro* chondrogenesis. The degree of post-transplantational suppression of mineralization of cartilage developed with PTHrP was not clear [75]. Intermittent administration of PTHrP(1–34) alleviated suppression of *COL2A1* [111]. However, the degree of suppression of *COL10A1* and *ALPL* expression by such treatment was marginal despite being statistically significant [111]. Since hedgehog stimulates the growth of

hMSCs and enhances their chondrogenesis to the *COL10A1*-expressing hypertrophic state [45, 112], suppression of hedgehog signaling by the PTHrP-activated PKA [113] may be one of the mechanisms by which PTHrP suppresses chondrogenesis from MSCs *in vitro*.

3.2. TGFβ-BMP signaling

TGFβ was first identified as cartilage-inducing factor (CIF-A) purified from bovine demineralized bone [114] but was soon found to have a negative effect on chondrocyte terminal maturation during scaffold-free 3-dimensional (3D) chondrogenesis culture (called "pellet culture") [115]. In fact, canonical TGFβ signaling that goes through the TGFβ receptor (TGFBR)–Smads such as Smad3 inhibits chondrocyte hypertrophic differentiation in the growth plate and, interestingly, also during *articular* cartilage formation [92, 116–118]. Furthermore, the TGFβ/Smad3 signaling system is essential for the integrity of developed articular cartilage [117, 119–124]. Therefore, TGFβ is now a standard ingredient for inducing chondrogenesis from MSCs [27, 125, 126]. BMP signaling is known to be essential for chondrogenesis *in vivo* [127, 128] and to enhance chondrogenesis from mesenchymal cells. It also facilitates hypertrophic differentiation of chondrocytes, although it delays terminal maturation of the hypertrophic chondrocytes [127, 129–132]. In combination, TGFβ and BMP signaling synergistically enhanced chondrogenesis [7, 133–136] but also stimulated the expression of signs of hypertrophic differentiation such as *COL10A1* transcript [133, 135, 137]. The latter effect was dependent on the type of BMP used (e.g., compared to BMP2, BMP7 is less likely to induce hypertrophic differentiation [138]). In support, activation of the BMP receptor (BMPR)–Smads (Smad 1/5/8) is linked to chondrocyte hypertrophic differentiation [73].

Thus, one way to stimulate chondrogenesis from chondroprogenitor cells while preventing hypertrophic differentiation of developed chondrocytes is to avoid overt activation of BMP signaling. Another is to select the appropriate BMP to use. For example, GDF5, one of the BMP-family proteins isolated from articular cartilage [139, 140], is involved in joint morphogenesis in the mouse [139, 141–145]. GDF5 is also needed for proper maintenance of articular cartilage in adult humans [146–149]. *In vitro*, GDF5 induces weaker chondrogenesis and chondrocyte hypertrophic differentiation than BMP4 during micromass culture of mouse limb bud mesenchymes [150] and suppresses expression of MMP13, one of the markers of terminally matured chondrocytes, during pellet culture of human chondrocytes [151]. Unfortunately, however, the use of TGFβ alone (i.e., without exogenous BMPs) for the induction of chondrogenesis *in vitro* is not guaranteed to result in immature, non-hypertrophic chondrocytes and often leads to the expression of hypertrophic chondrocyte markers [16, 74, 75, 138]. This effect could be due to the TGFβ–BMP synergy established by the TGFβ-induced production of BMPs from differentiating MSC, since TGFβ was found to induce BMP expression during growth plate chondrogenesis [152]. In fact, our results from mESC-derived mesodermal cells indicate that TGFβ-induced *in vitro* chondrogenesis depends on endogenous BMP activity [34]. In addition, TGFβ can activate the BMP signaling mechanism in chondrocytes: TGFβ normally signals through ALK5 (the Type I TGFBR) and TGFBR-Smad, but in the presence of CD105 (Endoglin), TGFβ signals through ALK1 (a Type I BMPR) and thereby activates BMPR–Smads instead [122]. Such a shift in the TGFβ signaling mechanism seems to

affect the stability and health of articular cartilage, which seems to be facilitated by WNT signaling [153].

Therefore, control of the strength and temporal action of BMP signaling via exogenously added BMPs such as GDF5 or BMP7 or by inhibitors for ALK1 or other BMPR–Smad activating receptors during chondrogenesis may lead to an optimal condition for hPSC-derived chondroprogenitors to form permanent cartilage preferentially. Alternatively, combinatory control with other signaling mechanisms such as WNT signaling may be needed.

3.3. WNT signaling

As noted, WNT signaling through the canonical pathway of stabilization and nuclear translocation of β-catenin stimulates osteogenesis and inhibits chondrogenesis by blocking *Sox9* expression [52] from osteochondrogenic mesenchymal cells during limb formation [154, 155]. However, canonical WNT signaling promotes hypertrophic differentiation of the chondrocytes typically in the growth plate [156, 157]. Genetic analysis has demonstrated that WNT/β-catenin signaling can initiate the hypertrophic differentiation process by inhibiting the PTHrP signaling mechanism [158]. One possible mechanism is via the direct binding of β-catenin to PTH1R, the major receptor for PTH and PTHrP [159]. Such physical interaction seems to switch the PTHrP–PTH1R signaling pathway from cAMP–PKA to Ca++–CaMKII, which is known to support the hypertrophic differentiation of chondrocytes (e.g., Mef2c-dependent expression of *Col10a1*). When the PTH1R-β-catenin interaction is blocked in chondrocytes, their hypertrophic differentiation is also blocked. Furthermore, WNT/β-catenin signaling stimulates terminal maturation of hypertrophic chondrocytes in a PTHrP signaling-independent manner, while it also indirectly stimulates BMP signaling [158]. Another possible mechanism would involve direct stimulation in chondrocytes of transcription of the hypertrophic differentiation-inducing genes such as *Ihh* [160], *Col10a1*, and *Runx2* [161]. In support, limb cartilage of *Wnt9a*^{-/-} mice express decreased levels of *Ihh* and *Col10a1* in hypertrophic chondrocytes [162, 163]. Interestingly, WNT/β-catenin signaling seems to help to establish the Ihh–PTHrP feedback loop. The *Wnt9a*^{-/-} limb cartilage also shows decreased levels of *Pthrp* at the ends of cartilage anlagen (e.g., the prospective shoulder joint). Thus, the canonical WNT signaling mechanism has a negative effect in the early stage of chondrogenesis and a positive effect in later stages, especially in the chondrocyte maturation stage, during endochondral ossification.

On the other hand, WNTs are implicated in the commitment of MSCs to differentiation. For example, WNT signals promote osteogenesis from MSCs by inducing *RUNX2* expression [164], which in turn induces expression of the osteogenic gene *SP7/OSX* [165]. In contrast, as with TGFβ signaling, WNT signaling prevents MSCs from adopting an adipogenic fate [166, 167]. The same holds true for the chondrogenic commitment of MSCs: WNT signaling inhibits chondrogenesis from hMSCs by actively suppressing the induction of Sox9 [40]. These observations suggest that the inhibition of canonical WNT signaling would promote chondrogenesis from MSCs and suppress hypertrophic differentiation of the developed chondrocytes. Recently, Narcisi et al. [50] reported the interesting observation that the suppression

of (all) WNT signaling during the last 3 weeks of the 5-week, serum-free, TGFβ-induced chondrogenesis from hMSCs not only suppressed, albeit marginally, signs of chondrocyte hypertrophic differentiation such as *COL10A1* and *ALPL* expression in the generated cartilage pellets but also maintained the pellets in an unmineralized state for 8 weeks at an ectopic site in nude mice.

3.4. Natriuretic peptide signaling

The C-type natriuretic peptide (CNP) signaling pathway is a major contributor to postnatal skeletal growth in humans [168, 169]. The CNP gene *Nppc* is highly expressed in chondrocytes in the growth plate [170, 171] and essential for bone formation [172–176]. The CNP receptor (membrane-bound guanylyl cyclase–coupled receptor B, *Npr2*) is a membrane-bound receptor carrying the intracellular guanylyl cyclase domain and is essential for eliciting skeletal phenotypes of CNP [177, 178]. CNP signaling thus results in upregulation of Cyclic guanosine monophosphate (cGMP) and activation of cGMP-dependent protein kinase (PKG). CNP stimulates hypertrophic differentiation of chondrocytes through activating the type II PKG (PKGII, *Prkg2*) [179, 180]. In fact, the phenotypes of mouse mutants of *Nppc*, *Npr2,* and *Prkg2* are similar [174, 177, 181]. Recently, glycogen synthase kinase (GSK 3β) was identified as the critical target of PKGII leading to the enhancement of β-catenin-dependent stimulation of hypertrophic differentiation of chondrocytes [182]. Thus, the CNP–NPR2–PKGII pathway can mimic canonical WNT signaling to stimulate hypertrophic differentiation of chondrocytes. However, manipulation of the CNP–NPR2–PKGII pathway during chondrogenesis from hMSCs or after the transplantation of hMSC-derived cartilage pellets for the generation and maintenance of permanent cartilage has not been tested extensively.

3.5. VEGF signaling

For tissue engineering approaches to cartilage repair, extensive improvements have been made to scaffold/hydrogel technology to provide a suitable chemical and physical environment for the embedded chondrocytes or chondroprogenitors. However, the growth factors tested to date have been selected for their potential to facilitate chondrogenesis (e.g., TGFβs or BMPs) rather than to prevent terminal maturation and mineralization. VEGF, the best known angiogenic factor, is produced in the growth plate specifically from hypertrophic chondrocytes, not from the resting or proliferating immature chondrocytes. Furthermore, VEGF-mediated blood vessel invasion is essential for coupling resorption of cartilage to bone formation [183]. Since the VEGFR2 (KDR/Flk1) is also expressed in hypertrophic chondrocytes, it is expected that the suppression of VEGF function may prevent terminal maturation and mineralization. The VEGF inhibition strategy has already been applied to stem cell-based cartilage regeneration therapy in animal models. Orthotopically transplanted MDSCs require BMP4 to differentiate effectively into chondrocytes to repair osteochondral defects in articular cartilage [4]. However, the suppression of VEGF function by forced expression of a soluble form of VEGFR1 (sFlt1) as well as BMP4 significantly enhanced the repair function of MDSCs [3, 5]. Incorporation of an inhibitor of VEGF signaling in a scafford embedded with human nasal chondrocytes allowed *in vivo* cartilage reformation after ectopic transplanta-

tion and increased the survival of the graft [184]. Thus, the manipulation of VEGF signaling may be useful in generating permanent cartilage. But thus far, any effect of suppression of VEGF action on the prevention of terminal maturation and mineralization of chondrocytes developed from hMSCs or (briefly cultured) chondrocytes *in vivo* has not been clearly demonstrated.

Bony cartilage recovered from transplanted mice, which originates from cartilage pellets generated by the hPSC-derived ectomesenchymal cells (**Figure 3** EctM), is usually highly vascular and becomes larger than the transplanted pellet (data not shown). In contrast, unmineralized or partially mineralized cartilage, which originates from cartilage pellets generated by uncultured paraxial mesoderm (**Figure 3** PM), is generally not vascular and slightly smaller than the transplanted pellet (data not shown). Suppression of production or function of VEGF from chondrocytes developed from adult- or hPSC-derived chondroproge-nitors may thus lead to long-lasting (i.e., permanent) cartilage *in vivo*.
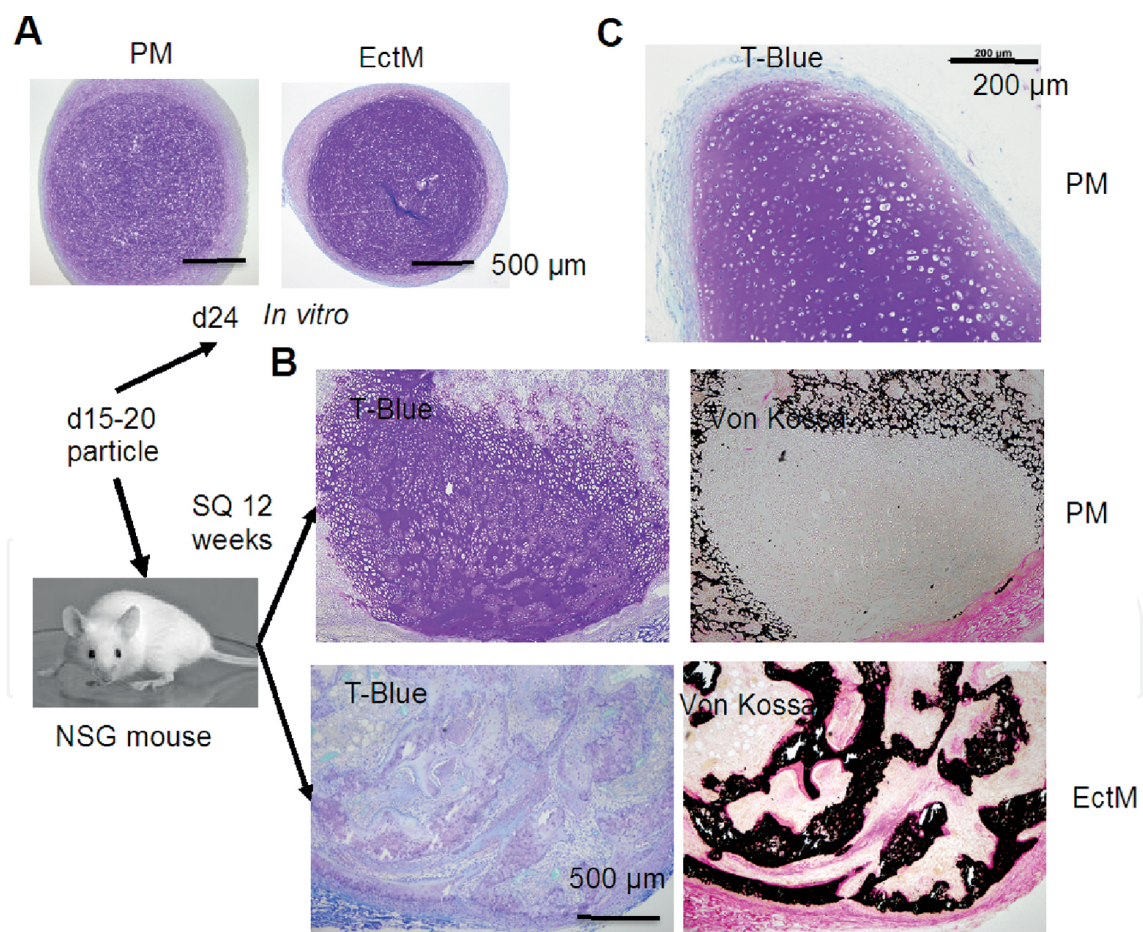
Figure 3. *In vivo* maturation of the cartilage particles developed with the FSB-expanded (p7) ectomesenchymal cells (EctM) and freshly isolated paraxial mesoderm (PM) derived from H9 hESCs. (A) The day-24 cartilage particles were directly fixed and stained with Toluidine Blue (T-Blue). (B) The day-20 cartilage pellets were transplanted into NSG mice subcutaneously (SQ) for 12 weeks, and were fixed, sectioned and stained with T-Blue and von Kossa. Paraxial

mesoderm-derived cartilage pellets constantly showed cartilaginous areas remain even after 12 weeks, and occasionally no sign of mineralization was detected (HES3 hESC-derived PM, C).

4. Cell types potentially leading to the genesis of permanent cartilage

The manipulation of signaling mechanisms may not be sufficient to generate permanent articular cartilage reproducibly *in vivo* or *in vitro*, even from hPSC-derived chondroprogenitor cells. Another strategy is to discover and use a novel population of cells derived from hPSCs that are themselves destined to form permanent joint cartilage or have the capacity to instruct other chondroprogenitors to form permanent articular cartilage. Permanent cartilage-forming cells have been sought in various adult tissues [185, 186]. The most relevant to articular cartilage regeneration would be synovial MSCs. However, thus far, no report has been published to indicate permanent cartilage formation from such MSCs [187]. Here, we will discuss potentially useful types of cells for articular cartilage regeneration that can be generated from hPSCs.

4.1. Nasal chondrocytes and their precursors (ectomesenchymal cells)-novel chondroprogenitor cells

Numerous attempts to regenerate permanent articular cartilage using chondrocytes from articular cartilage have had little success, mainly because of improper *ex vivo* expansion culture methods to compensate for their low cell yields. Nasal and auricular cartilage are permanent cartilage that develops from neural crest. Similarly to articular chondrocytes, nasal and auricular chondrocytes can be expanded in a medium containing 10% (v/v) FBS with TGFβ1, FGF2, and PDGF-BB, as demonstrated by Martin and colleagues [188]. Although inevitably dedifferentiated, the redifferentiation capacity of the cells was maintained at least for 2 passages, resulting in $COL2^{hi}COL1^{lo}$ hyaline cartilage pellets. The same group has explored the idea of using a tissue-engineering approach to repair articular cartilage by nasal chondrocytes [189]. Recently, they have demonstrated that human adult nasal chondrocytes can be clonally propagated as dedifferentiated cells with only a minimal loss of their re-differentiation capacity (i.e., ability to self-renew) under 5% (v/v) human serum with TGFβ1 and FGF2 [190]. The self-renewal activity is much stronger in nasal chondrocytes than in articular chondrocytes. Furthermore, cartilage constructs generated from dedifferentiated nasal chondrocytes showed a better capacity to repair condyle defects in goat articular cartilage than constructs made by dedifferentiated articular chondrocytes. Considering that adult stem cells generally require *ex vivo* expansion to a clinical scale for cartilage repair and regeneration, dedifferentiated nasal chondrocytes are a better cell source than articular chondrocytes and may even be better than MSCs.

On the other hand, the $CD271^+CD73^+$ ectomesenchymal cells expressing *SOX9* that can be expanded in CDM containing FGF2 and SB431542 for at least 16 passages develop from the MIXL1-GFP$^-$CD271^{hi}CD73$^-$ neural crest-like progeny of hPSCs (**Figure 1**) [31]. Therefore, $CD271^+CD73^+SOX9^+$ cells can be the developmental origin of nasal chondrocytes. However,

the type of cartilage formed and the optimal chondrogenesis-promoting culture condition for the CD271$^+$CD73$^+$SOX9$^+$ cells and dedifferentiated nasal chondrocytes are different (data not shown). Furthermore, cartilage pellets arising from the chondroprogenitors derived and expanded either from neural crest-like progeny (**Figure 3**) [31] or from paraxial mesoderm (data not shown) are unstable and readily mineralize in 8 weeks when transplanted subcutaneously into immunocompromized mice, as was found for adult MSCs [16, 74, 75]. By contrast, the unexpanded paraxial mesodermal progeny of hPSCs showed a greater capacity to generate hyaline cartilage particles *in vitro* than adult hMSCs [33], and the cartilage pellet made *in vitro* was stable for 12–19 weeks and showed only low levels of mineralization (**Figure 3**) [31]. Similarly, among adult cells, only unexpanded or briefly expanded articular chondrocytes retain the ability to regenerate long-lasting hyaline cartilage [74]. Such results suggest that current conditions for expanding chondrogenic mesenchymal cells whether from adult human tissues or hPSCs may skew the developmental fate of the cells upon induction of chondrogenesis toward growth plate-type chondrocytes. Since our protocol to generate chondrogenic ectomesenchymal cells involves expansion culture of neural crest-like progeny of hPSCs, we have been unable to determine whether expansion culture truly prevents the ectomesenchymal cells from generating nasal cartilage-like permanent cartilage.

When and how the decision is made during chondrogenesis to generate growth plate-like chondrocytes destined to form bone, or articular or nasal/auricular chondrocytes destined to form permanent cartilage are not yet fully understood. Research is undergoing to figure out the way to generate nasal chondrocytes from hPSC-neural crest-derived ectomesenchymal cells, which we believe will lead to answers to some of the questions.

4.2. Embryonic joint progenitor cells; articular chondroprogenitors or instructor cells for permanent cartilage formation

During embryogenesis, joint cartilage formation is initiated by the GDF5$^+$ joint progenitor cells, which are distinct from the "general (SOX9$^+$)" chondroprogenitors that can give rise to growth plate chondrocytes [191]. Lineage tracing experiments demonstrate that the GDF5$^+$ mesenchymal cells accumulate as a band within a cartilage anlage, constituting the interzone, or at the edges of the independently formed cartilage anlagen during joint (e.g., hip) formation [192, 193]. The joint progenitor cells expressing Wnt9a, Erg, and collagen IIA eventually give rise to articular cartilage, ligament, synovial lining, and other joint tissues but contribute little if at all to the growth plate cartilage. Isolated joint progenitor cells are induced to form chondroprogenitors, albeit weakly compared with the standard chondrocytes, in response to GDF5, and forced expression of Wnt9a during chondrogenesis is inhibitory. However, conditional deletion of β-catenin inhibited the genesis of lubricin-expressing, flat superficial zone-like cell layer on articular cartilage [192], suggesting that canonical WNT signaling may change the chondrogenic fate of the joint progenitor from mid-deep zone chondrocytes to superficial zone chondrocytes.

These results points to the potential usefulness of the joint progenitor cells for the repair of articular cartilage. Since the joint progenitors are characterized mainly during embryogenesis [191], it is conceivable to aim to generate, isolate, and characterize joint progenitor cells

from PSCs. There is an interesting observation suggesting that the suppression of hedgehog signaling might promote genesis of the GDF5+ cell during mESC differentiation [194]. As noted, hedgehog signaling, when not linked to the Ihh–PTHrP feedback mechanism, induces osteogenic gene expression in chondrocytes and facilitates endochondral ossification, which also causes osteoarthritic cartilage degradation in adult articular cartilage [195]. Unfortunately, simple suppression of hedgehog signaling does not promote the development of *in vivo* permanent cartilage from the chondrogenic mesoderm generated during ESC differentition even though at the level of gene expression it supports articular-type chondrocyte formation *in vitro*. [29, 30]. Continued efforts are warranted to identify and isolate (and expand as required) hPSC-derived GDF5+ cells to elucidate their role in developing articular cartilage and the repair of damaged articular cartilage.

4.3. Postnatal joint stem/progenitor cells?

Consistent with GDF5+ embryonic joint progenitor cells being involved in the formation of a superficial layer of articular cartilage [192], the superficial zone chondrocytes show a distinct pattern of expression of stem/progenitor cell markers (Notch1, Stro1, vascular cell adhesion-1, side population) [196–199]. Within the articular cartilage, Notch1 and Stro1 are expressed exclusively in the superficial zone, although Stro1 expression is not specific to articular cartilage, since the growth plate also abunduntly expresses Stro1 [200], and Notch1+ cells derived from the superficial zone are enriched in the colony-forming unit fibroblast (CFU-F) activity that is dependent on active Notch signaling [196]. Therefore, the superficial zone has been hypothesized to harbor stem/progenitor cell activity. However, it has not been demonstrated whether such stem/progenitor cells can behave as resident joint stem/progenitor cells and "regenerate or repair" articular cartilage more effectively than the conventional adult chondrogenic cells such as bone marrow MSCs.

Another area of potential interest with regard to joint stem/progenitor cells is the perichondrial groove of Ranvier (or the zone of Ranvier). The area is located at the periphery of growth plate and is enriched in proliferating cells. Studies by Karlsson et al. [200] on the knee of adult rabbits have proved the existence of different subpopulations of progenitor cells in articular cartilage and the perichondrial groove of Ranvier. As with the superficial zone chondrocytes of articular cartilage, the cells in the groove of Ranvier express markers associated with stem cells and their niche (e.g., Stro1 and Jagged1), whereas cells in the growth plate directly adjacent to it do not express many of such markers. Mice lacking Tgfbr2 signaling in developing limb bud mesenchyme fail to form interzone, resulting in the absence of interphalangeal joints [201] as well as tendons and ligaments [202]. The Tgfbr2-expressing cells are in fact first detected at embryonic day (E) 13.5 within the interphalangeal joint interzone. Interestingly, by E16.5, the Tgfbr2-expressing cells are enriched in the perichondrial groove of Ranvier, in part of the superficial layer of articular cartilage, in the synovium, and in the tendon's enthuses, and they remain in the same area postnatally [203]. Such Tgfbr2-expressing cells are slow-growing cells and exhibit stem cell traits in expressing joint progenitor markers.

The knowledge that joint progenitor-like cells, whether in the superficial zone of articular cartilage or the perichondrial groove of Ranvier, are present in postnatal joints, combined with

the establishment of molecular tools to detect them are likely to inform future studies on the biology of postnatal joint progenitor cells. Isolation and functional characterization of these joint progenitor candidates will not only open the possibilities for an alternative cell source for regenerative therapy for cartilage, but also for the discovery of specific small or large molecule therapeutics that facilitate the regenerative activity of endogenous joint progenitor cells. However, as far as cellular therapy or tissue engineering therapy is concerned, the limitations noted with autologous chondrocyte/MSC implantation will also apply to these stem/progenitor cell systems—they will require expansion to clinically relevant levels without the loss of their developmental potential.

5. Conclusion and future perspective

As expected, PSC-derived chondrogenic progeny has shown the advantage over adult tissue-derived MSCs of "expansion without loss of chondrogenic potential" (**Figure 1**). However, the permanent(-like) cartilage-forming activity residing in the uncultured paraxial mesodermal cells (**Figure 3** PM) and the nasal cartilage-forming activity that the ectomesenchymal cells should possess, appear not to be readily maintained during expansion, even under the defined CDM + FGF2 + SB431542 (FSB) condition. In this sense, there is still room for the improvement in the expansion culture system. Controlling hypertrophic differentiation during chondrogenesis from such expanded hPSC-progeny by manipulating known signaling mechanisms or using novel cell populations may overcome the problem associated with expansion culture. To date, however, such an approach either has not been systematically examined, even for the widely used adult stem cells, or has failed to give positive results. Probably, mechanistic principles that direct the generation of articular or nasal cartilage from corresponding precursor cells need to be elucidated for the reproducible generation of permanent cartilage from MSCs or hPSC-derived, expanded chondroprogenitors.

For many years, the major challenges to making the hPSC system practical for therapeutic purposes have been how to direct differentiation to the specific cell lineage of interest and isolate the target cell population. But the important next challenge will be to control the aging or maturation of cell types generated from hPSCs so as to increase their functionality when transplanted into host adult tissues. Lastly, we will discuss two challenges associated with the use of embryonic/fetal cells for adult tissue repair/regeneration, and their potential relevance to the repair of articular cartilage by hPSC-derived progeny.

5.1. Overgrowth of embryonic/fetal progenitors

Human PSCs (both ESCs and iPSCs) are teratoma-forming cells by definition. Therefore, contamination of "undifferentiated" teratoma-forming cells in the population of "differentiated, functional" progeny has been the major concern with the use of hPSC-derived progenitor cells for therapeutic purposes. Even though the standard hPSCs are what is called "primed" PSCs, that is, they show less ability to self-renew as a single cell than mPSC-like "naïve" PSCs, many strategies have been devised to remove undifferentiated hPSCs. The use of iPSCs is

associated with the additional concern that they are generated by transduction of adult tissue cells with a set of reprogramming genes that include *c-myc*—reactivation of the *c-myc* transgene after differentiation of iPSCs seems to cause spontaneous tumor formation [204]. New reprogramming methods that do not result in the integration of a stable transgene into the recipient chromosome were quickly developed [205, 206]. Thus, the technical issues associated with the PSC system are being resolved. However, there remains a definite "overgrowth" problem, common to primitive progenitor cells upon transplantation. Neural stem cells (NSCs) are the most frequently used PSC-derived progeny for transplantation experiments. The transplantation into the adult rodent brain of NSCs generated from hESCs and expanded in the presence of FGF2 and EGF resulted in long-term graft survival and neural stem-like behavior in the host striatum and subventricular zone [207]. However, NSCs expanded under Shh + FGF8 [208] as well as rosette-forming NSCs expandable under Shh + FGF8 or Shh + Notch-ligands [209], both of which originate from hESCs, have revealed signs of neural overgrowth upon transplantation as evidenced by large grafts composed of primitive neural progenitors/rosette structures. Thus far, the neural overgrowth problem has not been associated with simple contamination by undifferentiated PSCs in the NSC population. Furthermore, neural overgrowth directly from human basal forebrain precursor cells transplanted into rodent models of Huntington's disease has been reported [210]. Therefore, neural overgrowth seems to be associated generally with primitive neural progenitor cells, rather than PSC-derived progeny specifically, and the inhibition of Notch signaling seems to prevent such overgrowth [211].

High proliferative properties of embryonic/fetal cell types that are the main cell types derived *in vitro* from hPSCs may thus cause the problem of over-proliferation after transplantation. While the problem might be lineage/cell type dependent, we need to be aware of it when cartilage regeneration is performed using hPSC-derived chondroprogenitors and/or joint progenitors. Thus far, there have been no reports of cartilage overgrowth during the repair of articular cartilage by the use of PSC-derived progenitors.

5.2. Development of "adult"-type cells

As noted, the process of differentiation of PSCs in culture can mimic many aspects of early embryogenesis. However, the terminally differentiated, functional cell types derived from PSCs often represent the embryonic counterpart of the adult cell type of interest, suggesting that the *in vitro* differentiation of PSCs mimics the developmental processes of embryonic/fetal cell types, not necessarily those of the adult cell type. Unfortunately, the functionality of embryonic cells often differs from that of their adult counterpart. For example, it is well known that embryonic hematopoietic progenitor cells (HPCs) that first appear in the yolk sac during embryogenesis give rise to primitive erythrocytes expressing embryonic globins and also demonstrate poor marrow repopulation activity upon transplantation into the adult, partly because of their poor homing capacity to the marrow and their poor viability in circulation. *In vitro* development of HPCs from PSCs has been extensively studied. The capacity of PSCs to give rise to hematopoietic cells in culture was first demonstrated by Doetschman et al. [212] using mESCs. However, detailed analyses on the hematopoietic progenitors were not reported

until the early 1990s [213–218]. Since then, many have shown the development of myeloid cells, lymphocytes, and definitive erythrocytes expressing fetal (or sometimes adult) globins. Successful development of marrow-repopulating activity (MRA), the property of definitive hematopoietic stem cells (HSCs), from mPSCs has also been reported. However, the majority of such reports have not been followed up or reproduced. Thus far, the most reliable, reproducible, and widely used method of achieving the genesis of MRA involves forced expression of the HoxB4 [219–221] or Cdx4 [222, 223] transgene in differentiating mPSCs. No similar genetic methods have been reported for hPSCs [224]. Nevertheless, in order to make use of hPSCs for studying disorders of adult hematopoiesis or for marrow-reconstituting therapies, there is a need for a robust, reproducible way of generating from hPSCs HSCs that resemble those derived from cord blood and bone marrow, ideally without the need for genetic manipulation.

In addition, cardiac muscle cells and their precursors (i.e., cardiac stem/progenitor cells) have been developed from hPSCs [225, 226]. The integration of such cells into rodent heart muscle improved cardiac functions in rodent models of myocardial infarction [227–230]. However, as is the case for erythrocytes, embryonic and adult cardiomyocytes differ in the composition of their major functional protein, the myosin heavy chain (MHC). For example, in the mouse, the slow MHC (MHCβ or MYH6) is predominantly expressed in the ventricle of embryonic hearts, while fast MHC (MHCα or MYH7) is dominant in the adult heart. In humans, this switch in ventricular MHCs is less pronounced, and MHCβ persists in the adult ventricle. In addition, the spontaneous beating phenotype of the hPSC-derived cardiomyocytes resembles fetal but not adult cardiomyocytes, and the majority of the hPSC-derived cardiomyocytes fail to fully recapitulate the electrophysiological function of adult ventricular cardiomyocytes [225, 231]. The most critical limitation is their deficiency in I_{K1}, the potassium inwardly rectifying channel. Since the hPSC-derived, spontaneously beating, cardiomyocyte clusters can transiently serve as a pacemaker in a pig heart upon transplantation [227], their functional differences from adult cardiomyocytes may increase the risk of cardiac arrhythmias upon transplantation. Therefore, similar to the case of HSCs, a robust, reproducible method that generates from hPSCs cardiac stem/progenitor cells capable of giving rise to mature adult cardiomyocytes is needed if hPSCs are to be used for the repair of damaged heart muscle.

Where cartilage is concerned, no adult–fetal differences in the biochemical property of joint chondrocytes have been elucidated. Differences have only been observed in the cellularity and physical properties of child and adult articular cartilage. Since there appears to be no active replenishing of articular chondrocytes or spontaneous regeneration of damaged articular or meniscus cartilage in adult humans and large animals, we hypothesize that embryonic/fetal cells are likely to perform better in cartilage repair than adult cells.

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