Lessons from joint development for cartilage repair in the clinic

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Abstract
More than 250 years ago, William Hunter stated that when cartilage is destroyed it never recovers. In the last 20 years, the understanding of the mechanisms that lead to joint formation and the knowledge that some of these mechanisms are reactivated in the homeostatic responses of cartilage to injury has offered an unprecedented therapeutic opportunity to achieve cartilage regeneration. Very large investments in ambitious clinical trials are finally revealing that, although we do not have perfect medicines yet, disease modification is a feasible possibility for human osteoarthritis.

KEYWORDS
cartilage regeneration, injury, joint development, osteoarthritis, stem cells, WNTs

1 DEVELOPMENTAL MORPHOGENESIS AND HOMEOSTATIC RESPONSES TO INJURY SHARE BASIC MECHANISMS

During embryonic development, mesenchymal cells deriving from the lateral plate mesoderm (LPM), condense within the limb bud to form a cartilage anlage. The chondrocytes in the center (diaphysis) of the skeletal elements undergo hypertrophic differentiation, expressing collagen type X, VEGF, MMP13, and alkaline phosphatase. Hypertrophic differentiation, in turn, triggers mineralization and ultimately replacement by bone.1–3 Endochondral ossification proceeds from the diaphysis towards the epiphysis sparing the joint interzones, which separate the future skeletal elements and will give rise to the joints. The joint interzones are composed of a distinct population of chondrogenic precursors expressing GDF5, WNT9A, WNT16, and other markers. Sox9+ cells from the limb mesenchyme are recruited to the joint interzone where they transiently express Gdf5.4 Cavitation through the middle of the joint interzone completes the separation of the skeletal elements. The first cells recruited to the Gdf5+ interzone

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lineage contribute largely to the epiphyses; cells recruited at later stages contribute to the articular cartilage and finally to the other soft tissues of the joints, including menisci, synovial membrane, ligaments and tendons.\textsuperscript{3,5-7} WNT signaling is required and sufficient for joint formation. While the deletion of individual genes results in minor phenotypes—for instance, deletion of \textit{Wnt9a} results in synovial chondromatosis,\textsuperscript{8} possibly because of compensation—complete suppression of WNT signaling resulted in joint fusion.\textsuperscript{9,10} Misexpression of \textit{Wnt9a} was sufficient to induce interzone markers including \textit{Gdf5}, but not the formation of ectopic joints.\textsuperscript{10} \textit{Gdf5}-null mice lack some distal skeletal elements in the autopod, and also have anomalies in the proximal joints: for instance, the knees are missing the menisci and the cruciate ligaments.\textsuperscript{7,11} The more severe phenotype in the distal skeletal elements is possibly due to the proximo-distal expression gradient of \textit{Gdf5} compared to other BMPs which may partially compensate in the proximal joints. Deletion of \textit{c-Jun}, which is upstream of \textit{Wnt9a} in the joint interzones, also resulted in lack of joint cavitation,\textsuperscript{12} however, morphologically, joint interzones were still present both after WNT blockade and \textit{c-Jun} deletion.\textsuperscript{9,12} Therefore, while substantial progress has been made in the understanding of joint morphogenesis and maturation, the prime mechanisms determining joint specification remain elusive.

As opposed to the epiphyseal cartilage, the articular cartilage is stable throughout life, resistant to hypertrophic differentiation and endochondral ossification. Once the skeleton is mature, in adult life, the articular cartilage enters a status of extremely low turnover.\textsuperscript{13} However, shortly after injury the articular cartilage deploys an impressively rapid homeostatic response that, in many cases, restores integrity and function.\textsuperscript{14} This repair response is triggered by the re-activation of several of those molecules which during development mark the joint interzone and are not normally expressed in quiescent uninjured adult cartilage.\textsuperscript{15} These responses contribute to healing\textsuperscript{16} and to re-establishing homeostasis.\textsuperscript{17,18} Spontaneous healing is most likely to be successful for small isolated lesions in otherwise healthy and young patients. With age, joint instability and other comorbidities including obesity, the likelihood of successful repair decreases.\textsuperscript{14}

If repair is unsuccessful, or if injury persists, the excessive or prolonged molecular responses to injury may become a pathogenic factor leading to ectopic activation of hypertrophic differentiation and mineralization within the articular cartilage. This, in turn, drives further cartilage loss and development of osteoarthritis.\textsuperscript{19-21} Osteoarthritis is the most common cause of disability for which we have no cure.\textsuperscript{22} Targeting basic mechanisms shared by developmental morphogenesis and injury responses has allowed researchers to aim for cartilage regeneration for disease modification (Figure 1). One key example of this is ectopic chondrocyte hypertrophy: while hypertrophic differentiation is essential for normal development of the appendicular skeleton, adult articular chondrocytes should not undergo hypertrophy. When homeostasis is disrupted, a series of events requiring the transcription factor HIF-2\textalpha{} induce ectopic hypertrophy within the articular cartilage; the articular cartilage becomes mineralized and further cartilage loss ensues\textsuperscript{19-21} (Figure 2). Indeed, genetic deletion of \textit{Hif-2\textalpha{}} in adult cartilage protected chondrocytes from ectopic hypertrophic differentiation and resulted in decreased cartilage degradation in murine models of osteoarthritis.\textsuperscript{20,21} Additional mechanisms shared between developmental morphogenesis and homeostatic responses to injury are shown in Figure 1 and summarized in Table 1.

At this point the main questions were: how can we utilize such mechanisms to support cartilage repair? What are the molecules that, in injured adult cartilage, become reactivated and regulate these processes? What initiates production of stable cartilage, which does not result in bone formation? A transcriptomic analysis of adult articular cartilage, 24 hours after injury, revealed the re-expression of morphogenetic signals that play important roles during joint development but are otherwise inactive in the adult cartilage including the WNT, TGF-\beta/BMP, Hedgehog, and FGF signaling pathways.\textsuperscript{15,16,41}

The reactivation of such molecules in the acute-injury phase has long-term consequences on the outcome of repair. The focus on early morphogenetic events is a complete change of perspective compared to the previous emphasis, in osteoarthritis research, which targets late, downstream events such as extracellular matrix production and degradation. Two examples are the reactivation of \textit{Wnt16} and \textit{Pthr1/PThrP} in adult cartilage after injury.

### 1.1 The case of \textit{Wnt16}

\textit{Wnt16} is one of the earliest markers of the joint interzone,\textsuperscript{9,10} but is undetectable in adult cartilage.\textsuperscript{15,17} We showed that 1 day after injury, \textit{Wnt16} became abundantly re-expressed in cartilage and canonical WNT signaling became activated.\textsuperscript{15} The re-expression of \textit{Wnt16} after injury was transient, however adult mice lacking \textit{Wnt16} developed more severe cartilage loss and osteoarthritis 8 weeks following joint destabilization.\textsuperscript{17} During its injury-induced expression, \textit{Wnt16} supported the maintenance and activity of a \textit{Prg4+} stem cell population within the superficial layer of the articular cartilage.\textsuperscript{42,43}
while at the same time preventing more potent WNT agonists from uncontrollably activating the β-catenin-dependent pathway, which would lead to ectopic cartilage hypertrophy and ultimately loss of cartilage. Tong et al. showed that Wnt16 overexpression protected cartilage in a model of osteoarthritis. Wnt16 delayed cartilage breakdown by activating JNK and the WNT planar cell polarity pathway, thereby ultimately up-regulating PTHrP expression.

1.2 | PTHrP/PTH1 signaling

In the periarticular cartilage of the developing skeleton, activation of parathyroid hormone receptor 1 (PTH1) by parathyroid hormone-related protein (PTHrP) increases proliferation and inhibits ectopic hypertrophic differentiation. This mechanism allows elongation of the bones. PTH1 is not normally expressed in adult articular cartilage. After chronic injury, such as in osteoarthritis, PTH1 was re-expressed and, when stimulated by exogenous recombinant human parathyroid hormone, it protected cartilage from breakdown.

2 | THE PATHWAY TOWARDS DISEASE MODIFICATION AND CLINICAL APPLICATION

Manipulation of the molecules driving the homeostatic responses to cartilage injury has led to remarkable results in animal models, with a seemingly endless list of potential targets. In practice, however, several factors limit clinical applicability. Some molecules play important roles in several tissues and targeting these could possibly lead to undesired effects. For instance, this is the case of mTOR, which is a central regulator of metabolism. The mTOR inhibitor rapamycin improved the outcome of osteoarthritis in mice, but it has severe side effects on overall health. Accessibility is also a limiting factor. Intracellular molecules and transcription factors are often difficult to target in humans. The route of delivery and pharmacokinetics also present limitations: for instance, intra-articular injections may limit systemic toxicity, but they are painful and would not be tolerated by patients if required too frequently.

Hereafter we review, using three examples, the path that has led to clinical experimentation and, in some cases, successful clinical trials.

2.1 | Blockade of WNT signaling

WNTs are a family of secreted morphogens originally discovered for their role in oncogenesis and subsequently well studied for their role in embryonic morphogenesis. In the absence of WNTs, β-catenin is constitutively degraded. When so-called “canonical” WNTs such as WNT1 or WNT3A bind to frizzled (FZD) receptors and to their co-receptors LRP5 and LRP6, the molecular complex responsible for β-catenin degradation is disrupted. This causes β-catenin to accumulate in the cytoplasm, translocate to the nucleus where it binds to TCF/LEF transcription factors and contributes to the activation of target genes. Other WNTs activate pathways collectively denominated “non-canonical,” mediated by different co-receptors including ROR1 and ROR2.

Early cell and developmental biology experiments showed that the canonical WNT pathway regulates skeletogenesis and joint morphogenesis.
broad terms, these studies suggested that activation of canonical WNT signaling inhibited chondrogenesis in progenitor cells and initiated hypertrophic differentiation in mature chondrocytes.24

Wnt4, Wnt9A, and Wnt16 are the earliest markers of joint interzones.9,10 Although WNT signaling is essential for joint formation,9,10,55-57 the specific functions of individual ligands and receptors are largely redundant. Their function in development and adulthood are summarized in Table 2.

In adult cartilage, both inhibition70 and forced activation of β-catenin signalling73 led to cartilage destruction in mice. This is because a population of cartilage-specific chondroprogenitors expressing Prx442 is dependent on β-catenin signalling73 whereas excessive WNT activation inhibits chondrogenesis and drives hypertrophy in already differentiated chondrocytes.9,24,37,56

Consequently, a “Goldilocks” theory took hold whereby WNT activation needs to be above a certain level in order to maintain progenitor cell populations, but below levels which drive hypertrophic differentiation in mature chondrocytes. Further complexity was added by the understanding that while a short burst of WNT activation supports articular cartilage formation and homeostasis,17,57 excessively prolonged activation resulted in cartilage breakdown. Interestingly, WNT16 is both required and sufficient for cartilage homeostasis following cartilage injury,17,44 and it is a partial activator of the canonical WNT pathway, maintaining “homeostatic levels” and preventing excessive activation from other more potent ligands.17,44

The relevance of these findings to human osteoarthritis was confirmed by the association of allelic variants of WNT inhibitory molecules such as FRZB74 and DOT1L75 with osteoarthritis. These data were replicated in animal models.28,54

These exciting data triggered the search for WNT inhibitors which could be used to treat osteoarthritis. Given the high level of redundancy of WNT ligands and receptors, it was unlikely that WNT inhibition outside or at the level of the cell membrane would be successful. Inhibition of WNT signaling through up-regulating FRZB, by Verapamil,76 or with a small molecule XAV-93977 led to improved outcomes of osteoarthritis in animal models.

Deshmukh et al. identified a small compound (SM04690, now commercialized by SAMUMED as Lorecivivint) which, by inhibiting the intracellular kinases CLK2 and DYRK1A, inhibited WNT signaling downstream of β-catenin.78,79 SM04690 proved to have a remarkable pharmacokinetic profile: after intra-articular administration in rats it could not be detected in plasma, it was detected just above therapeutic levels in bone, but it accumulated in cartilage for at least 180 days.79 A single intra-articular administration of SM04690 improved structural outcomes in an instability-induced osteoarthritis model and improved structural outcomes, pain and weight-bearing in the monosodium iodoacetate model in rats.79 In a phase I clinical trial, a single intra-articular injection of SM04690 proved to be safe after 1 year follow-up in patients with osteoarthritis (Kellgren-Lawrence score 2-3). Although the study was not designed for, and was vastly underpowered to test efficacy (61 patients in total followed up for 1 year), pain parameters were improved at all doses and joint space width was improved in the intermediate dose.80 A phase II study, although it did not meet its primary endpoint,
confirmed pain relief and some evidence of improvement of joint space width in patients with unilateral osteoarthritis at an intermediate dose. Interestingly, and in keeping with the notion of a "Goldilocks zone" of WNT activation, the intermediate dose yielded the best results. Whether the long-term efficacy of this compound after a single injection is due to its long-term accumulation in cartilage or that perhaps the initial delivery is sufficient to trigger a self-maintaining homeostatic cascade is unknown. Although these results are promising, phase III studies are required to demonstrate efficacy.

One important contribution of this article was the improvement of the clinical trial technology in osteoarthritis. In particular, the finding that selecting patients with unilateral symptoms and without widespread pain allows a higher level of sensitivity, especially in terms of pain, will greatly facilitate future studies with this or other compounds.

### 2.2 TGF-β and bone morphogenetic proteins

In 1965 Urist and Daly published the seminal paper "Bone: formation by autoinduction" in which they described that ectopic implantation of demineralized bone matrix triggered ectopic endochondral bone formation, ultimately leading to the formation of an ossicle made by cells originating from the host organism. They also correctly hypothesized that some substance contained in the acellular bone matrix would be responsible for initiating the morphogenetic events leading to bone formation. In the years that followed, several groups went on to identify a family of molecules, named Bone Morphogenetic Proteins (BMPs), which, when implanted in an appropriate matrix, resulted in ectopic cartilage and bone formation. It became apparent that, far from being specific cartilage/bone growth factors, BMPs had a broad array of functions in different cells, stages of development and adult life. Regulation of the BMP pathway is essential for gastrulation, the formation of mesenchyme in early development, establishing the dorso/ventral patterning and the morphogenesis of virtually all organs.

In spite of relatively stereotypical, shared signaling mechanisms and similar in vitro effects on skeletal cells, the expression pattern and the biological activity of different BMPs in vivo varies enormously. This is exemplified by the elegant studies in the chick model in the Hurle laboratory in the mid-1990s. Macias et al. showed that BMP7 is expressed in the diaphyseal perichondrium, skipping the joint interzone, and when delivered next to a joint interzone, it inhibited joint formation. Conversely, BMP2 was expressed in the joint interzone and its ectopic delivery resulted in ectopic joint-like structures.

Because of the capacity of BMPs to induce cartilage and bone formation, several products were generated and tested for the repair of critical size defects, non-unions and spinal fusions, however, over 40 years after the discovery of BMPs, the results of clinical testing in osteoarthritis and chondral defects are underwhelming. This could be due to several factors, but the stability of these molecules in inflammatory sites, their pleomorphic function in different settings and different cells, and especially their propensity to induce ectopic cartilage and bone represent serious issues. Overexpression of BMP2 in adult joints resulted in ectopic cartilage and bone formation within the joint soft tissues and additional cartilage degradation through up-regulation of matrix metalloproteinases.

To circumvent this problem, investigators have tested the use of transforming growth factor (TGF)-β. TGF-β is a potent inducer of chondrocyte differentiation in a variety of stem cells, promoting SOX9 expression, extracellular matrix production.

In humans, a genetic association of alleles of TGF-β and downstream molecules with osteoarthritis was identified. In keeping with this, transgenic expression of a dominant negative Tgf-β receptor 2 (Tgf-βr2) led to
formation of hypertrophic cartilage and an osteoarthritis-like phenotype. However, in vivo, TGF-β overexpression exacerbated osteoarthritis, whereas its inhibition was beneficial in murine models. This was true when using the TGF-β inhibitor halofuginone, an anti-TGF-β1, systemic delivery of a TGF-βR1 inhibitor or by knocking out Tgf-βr2 in nestin-positive MSCs.

The discrepancy between the requirement of TGF-β signaling in joint homeostasis and its negative effect when overexpressed in osteoarthritis remains enigmatic. One explanation was offered by Blaney-Davidson et al. who demonstrated that up-regulation of ALK1 receptor switches TGF-β signaling from SMAD2/3 which inhibits chondrocyte hypertrophy to SMAD1/5/8 downstream, which induces chondrocyte hypertrophy.

### Table 2: Roles of WNTs in cartilage development and adulthood

| Molecule | Development | Adult cartilage |
|----------|-------------|-----------------|
| WNT3A    | Maintains mesenchymal stem cells in an immature state in the developing limb bud, in chicks induces expression of FGF8 in the apical ectodermal ridge, promoting its formation. | Dose-dependent activation of β-catenin pathway, increasing chondrocyte proliferation; and non-canonical Ca²⁺-, CaMKII dependent pathways leading to dedifferentiation of chondrocytes. |
| WNT4     | Expressed at site of future joint formation. Promotes chondrocyte differentiation and hypertrophy, regulating the transition from pre-hypertrophy to hypertrophy, but is also not strictly required for normal joint formation. | Synergizes with WNT9A in preventing ectopic chondrogenic differentiation of synovial MSCs. |
| WNT5A    | Expressed in the perichondrium and regulates limb outgrowth through the planar cell polarity pathway, by activating ROR2 and Vangl2. Inhibits chondrocyte transition from a resting state to proliferative. It is required for the transition from proliferative to prehypertrophic state, but blocks hypertrophy. Knockout and overexpression of WNT5A show similar phenotypes in vivo. | Reduces COL2A1 and Aggrecan, and induces MMP1 and MMP13 expression in osteoarthritic chondrocytes. |
| WNT8     | Expressed in the perichondrium. Promotes chondrocyte hypertrophy and calcification. | |
| WNT9A    | Expressed at site of future joint formation. Blocks and can reverse chondrocyte differentiation. Ectopic expression in chick limbs induces ectopic joint interzone formation—but not required for joint formation. | Wnt9A deficient mice develop synovial chondromatosis. |
| WNT16    | Early marker of joint interzone. Prevents chondrocyte hypertrophy. | Buffers activation of β-catenin pathway by stronger canonical WNT ligands. Promotes cartilage homeostasis by maintaining Prg4+ expression, and prevents hypertrophy through regulating mTORC1-PTHrP. |
| Inactivation of β-catenin | Joint fusion and delayed endochondral ossification. Reduced lubricin and COL2A1 expression. | Increased proteoglycan content and reduction in endochondral ossification in vitro. Increased chondrocyte apoptosis and articular cartilage destruction in vivo. |
| Over-activation of β-catenin signaling | Blocks and can reverse chondrocyte differentiation. Ectopic expression of constitutively active β-catenin in chick limbs induced ectopic joint interzone formation. | Promotes chondrocyte differentiation, hypertrophy and cartilage calcification. Catabolism of cartilage extracellular matrix and chondrocyte apoptosis. Worse outcomes in rodent OA models with cartilage loss and osteophyte formation. |
The disappointing results of the use of TGF-β and BMPs in cartilage repair can be attributed to the fact that although BMP and TGF-β signaling are essential for cartilage morphogenesis and homeostasis, their function is tightly regulated both spatially and temporally, and inappropriate or excessive activation are detrimental.

### 2.3 | FGF18

Fibroblast growth factor (FGF) signaling was one of the first pathways discovered to be activated by cartilage injury. It contributes to repair responses through release of FGF2 from the injured articular cartilage.106,107 However, FGF2 activates both FGF receptor-1 (FGFR1) and FGFR3, the former associated with prevalently catabolic effects on articular cartilage and the latter promoting anabolic events.108,109

Mutations of FGF receptors result in a variety of skeletal defects.110 With few exceptions, mutations of FGFR1 and FGFR2 result in defects of skeletal elements that form through intramembranous bone formation (craniosynostoses and similar syndromes); whereas mutations in FGFR3 result in dwarfsisms caused by defects of the bones that form through endochondral bone formation. For all these reasons, FGFR3 signaling was considered a suitable target for articular cartilage homeostasis. Dominant activating mutations in FGFR3 resulted in hypochondroplasia, achondroplasia111 and thanatophoric dysplasia.112 Recessive loss-of-function mutations of FGFR3 resulted in camptodactyly, tall stature, scoliosis, and hearing loss syndrome (CATSHL syndrome).113 Mice lacking Fgfr3114,115 had features resembling CATSHL syndrome, whereas mice with activating mutations of Fgfr3 had features similar to achondroplasia.116 The skeletal defects were due to a delay in chondrocyte hypertrophy and endochondral bone formation. Given the pathogenic role of ectopic chondrocyte hypertrophy in the adult articular cartilage, this property of FGFR3 signaling supported its activation to prevent osteoarthritis progression.

In addition to halting chondrocyte hypertrophy and endochondral bone formation, FGFR3 is essential for the differentiation of mesenchymal cells into chondrocytes, as limb bud mesenchymal cells from Fgfr3 knockout mice failed to undergo chondrogenesis in 3D culture and to proliferate in monolayer.117 Finally, the expression of FGFR3 is associated with the capacity of adult articular chondrocytes to form stable articular-like cartilage in vivo108 and to repair cartilage defects in goats118 and humans.119,120

Activating FGF receptors only in cartilage is difficult because FGFs are ubiquitous, pleiotropic and several FGF ligands can signal through multiple FGFRs. Fortunately, FGFR3 is mostly expressed in cartilage, its mutations result almost exclusively in skeletal phenotypes. FGF18, a selective ligand for FGFR3, promoted chondrocyte proliferation and differentiation.121,122 Alleles of FGF18 were genetically associated with osteoarthritis in humans.100 Therefore, recombinant FGF18 was tested as a therapeutic for osteoarthritis. Moore et al. showed that intra-articular injections of recombinant FGF18 induced chondrocyte proliferation and cartilage repair when delivered in a therapeutic regime in rats subjected to a severe model of instability-induced osteoarthritis.122

Two large clinical trials provided evidence that treatment with intra-articular recombinant FGF18 (developed by Merck and Nordic Bioscience as Sprifermin) resulted in some degree of improvement of cartilage integrity in osteoarthritis.123,124 However, symptomatic improvement which was suggested in the first trial (phase I)124 was not replicated in the subsequent larger trial (phase II).123 Clearly, pain is a fundamental outcome for patients. We do not know whether the failure to detect pain improvement with FGF18 was due to trial design, for instance by not excluding patients with widespread chronic pain, or whether FGF18 treatment does not result in pain relief. Published pre-clinical data in animal models did not include pain measurements.122 A corollary is that, in the absence of pain relief, it is difficult to judge the clinical relevance of the small degree of improvement in joint space narrowing reported by the authors.

Taken together, data on inhibition of WNT signaling and using FGF18 suggest that targeting homeostatic pathways has led us to turn the corner in developing pharmacological approaches for treating osteoarthritis. It is likely that improvements in clinical trial design and patient stratification will be key to measure efficacy.

### 3 | FROM CELL-BASED THERAPEUTICS TO STEM CELL NICHEs

In the mid-1990s, Brittberg et al. successfully repaired full thickness cartilage defects by implanting autologous chondrocytes that had been briefly expanded in vitro.125 Since then, autologous chondrocyte implantation (ACI) has been tested in multiple clinical trials and has resulted in good and persistent clinical and structural benefits.126,127 Unfortunately, due to the autologous nature of the cells, these technologies are extremely laborious, costly, are not easily upscalable and therefore have not reached routine clinical application.
Transplantation of mesenchymal stem cells (MSCs) of various origins into cartilage defects has also shown some degree of efficacy, although the clinical trials so far have been much smaller than those with chondrocytes. Stem cells are clonogenic cells that have two remarkable features: the ability to differentiate into multiple lineages (multipotency) and the ability to simultaneously replenish the stem cell pool (self-renewal). Bone marrow mesenchymal cells, the first adult MSCs identified, were originally discovered as plastic adherent, non-haematopoietic clonogenic cells that can differentiate into multiple skeletal lineages including adipogenic, osteogenic, and chondrogenic.

The subsequent discovery that multiple stem cell populations persist within adult skeletal tissues opened up the possibility of harnessing the regenerative machinery of the adult joint by recruiting local resident stem cells to the site of damage. Stem cells originating from different tissues—such as the synovial membrane, the periosteum, the bone marrow, and the cartilage itself—have distinct “default” differentiation pathways. The development of lineage tracking technologies enabled a much more sophisticated understanding of the nature and function of different progenitor lineages in development and in post-natal repair.

The pressing questions in order to pursue this opportunity are: why are there so many different stem cell types? Do they differ in their repair capacity? What are the molecules that govern their niches?

3.1 | Progenitor lineages within the developing limb bud persist and contribute to healing in the adult skeleton

The development of the appendicular skeletal elements requires the proliferation and migration of mesenchymal cells from the LPM to form the limb bud. The MSCs at the periphery of the limb bud are maintained in an undifferentiated state by molecular signals (specifically FGF8 and WNT3A) which are released from the apical ectodermal ridge (AER) and surrounding ectoderm respectively. However, in the center of the limb bud, away from the control of these signals, MSCs aggregate into the mesenchymal condensations to form the skeletal anlage. From the early stages of limb development, the anlage appears to be composed of distinct cell populations which give rise to specific cell lineages and sub-lineages, and ultimately to different skeletal structures (Figure 3).

3.1.1 | PRX1+ progenitors

Paired Mesoderm Homebox 1 (PRX1) is one of the first progenitor cell markers that appears in the limb bud. PRX1 is expressed as early as 9.5 days post coitum (dpc), stemming directly from the LPM, and by 10dpc it can be detected in almost all the skeletal mesenchyme in the limb, including the condensing mesenchyme, chondrocytes, periosteum and tendons. By 15dpc however, the expression of PRX1 becomes restricted to the periosteum and tendons. The PRX1-expressing periosteal cells are maintained after birth and retain their capacity to differentiate into chondrocytes in vitro, suggesting that these periosteum-PRX1+ cells are both chondro- and osteo-progenitor cells.

The generation of Prx1-cre mice and their crossing to suitable reporters have demonstrated that PRX1-lineage progenitor cells give rise to all the cell sub-lineages that contribute to the formation of the bony elements, the articular cartilage, tendons and ligaments. PRX1+ cells also persist as MSCs postnatally within the periosteum where they contribute to cartilage repair.

The PRX1 lineage arises from a still earlier mesenchymal lineage expressing platelet-derived growth factor receptor α (Pdgfra) which appear as early as 6.5 dpc. Interestingly, Pdgfra is also a marker of progenitor cells which persist within the adult synovial membrane, and that contribute to cartilage repair.

Within the PRX1+ population, two distinct MSC populations emerge. Osterix + cells give rise to calcified tissues including bone and the calcified layer of the articular cartilage; and Gdf5+ cells which give rise to the articular cartilage and other soft tissues of the joints including ligaments and tendons.

3.1.2 | Osterix+ progenitors

During embryonic development, Osterix (Osx) is expressed by both the epiphyseal chondrocytes where it promotes hypertrophic differentiation and by an MSC population residing around the vessels of the perichondrium. Subsequently, the Osx + periosteal precursors enter the hypertrophic cartilage accompanying the ingrowing vessels and give rise to osteoblasts to form the bone replacing the hypertrophic cartilage.

Multiple separate cell lineages were detected within developing epiphyses including Gremlin+, Nestin+, and LeptinR+ progenitor cells. Whereas their identity, marker profile, and differentiation potential has been well-studied in embryonic development, their...
persistence as distinct progenitor populations in adulthood and particularly their contribution to tissue repair is unclear. For instance, Nestin+ cells persist in the peri-vascular spaces of the periosteum and in the adult synovial cells, however, in the absence of proper lineage tracking, their respective contribution to fracture and cartilage repair remains unconfirmed.

3.1.3 | Scx+SOX9+ progenitors

Progenitor cells which contribute to the formation of tendons and their attachment to bones (entheses) express Scleraxis (Scx) and Sox9. Scx was required for the formation of tendon-to-bone attachment sites. TGF-β and BMP4 signaling were essential for the specification and differentiation of Scx+Sox9+ progenitors, respectively. Further analysis was able to dissect another distinct subset of progenitors from this pool, those which are Scx+Sox9−; both Scx+Sox9+ and Scx+Sox9− progenitors can differentiate into tenocytes, however, those closest to the cartilaginous primordium are mostly derived from the Sox9+ population. The Scx+Sox9+ lineage persist in the adult enthesis and periosteum and contribute to tendon and fracture healing.

3.2 | Joint progenitors

The cells that form the adult articular cartilage derive from two main lineages: the superficial and intermediate zone are contributed from progenitor cells deriving from


Gdf5+ progenitors,\textsuperscript{5,42,43} whereas the deep layer, which is calcified, is contributed and renewed by Otx+ progenitors\textsuperscript{42} and therefore can be considered a remnant of the epiphyseal growth plate.

3.2.1 | GDF5+ progenitors

During embryonic development, Gdf5/Cdmp1 is expressed within the portion of the cartilage anlage that forms the embryonic skeleton and is destined to give rise to the permanent articular cartilage. Lineage tracking experiments showed that all soft tissues (such as the articular cartilage, meniscus, synovial lining, and joint capsule) within the joint are composed of cells that, within the interzone, derive from the Gdf5+ progenitors.\textsuperscript{5-7} Until recently, Gdf5 lineage cells were thought to be determined early during embryonic development and represented a stable population. Challenging this hypothesis, Schwartz et al. elegantly demonstrated that there is a constant inflow of Gdf5-lineage cells recruited to the joint interzone throughout development from the surrounding Sox9+ mesenchyme and, depending on the time when such recruitment occurs, they contribute to different joint structures: the first cells to be recruited contribute to the epiphyses, then to the articular cartilage and the last ones to menisci and cruciate ligaments.\textsuperscript{7} In addition, these studies demonstrated that far from being a “stable marker,” Gdf5 expression in the interzone cells is transient.

Gdf5-lineage cells have recently been shown to persist in the adult joint in the perivascular spaces of the synovial membrane and to contribute to the repair of cartilage injuries.\textsuperscript{37,38}

3.2.2 | LGR5+ progenitors

A subpopulation of Gdf5 lineage progenitors acquire the expression of Lgr5 and Col22a1 and give rise specifically to cruciate ligaments, synovial membrane, and articular chondrocytes.\textsuperscript{171} LGR5 is a receptor that amplifies the effect of β-catenin dependent WNT signaling through binding R-spondins,\textsuperscript{172} a signaling pathway that the progenitor cells of the superficial cartilage layer are strictly dependent on.\textsuperscript{43} Implantation of embryonic Lgr5+ cells into an adult murine cartilage defect resulted in cartilage repair,\textsuperscript{171} however it is not known if Lgr5+ cells persist in the adult joint and, if so, whether they contribute to cartilage healing.

Interestingly, however, Lgr5 expression is detected in the Gdf5-lineage population, prior to the expression of Prg4,\textsuperscript{171} an additional chondro-progenitor which largely resides in the surface of the articular cartilage.

3.2.3 | Prg4+ progenitors

Prg4 (the gene encoding lubricin) is expressed in the mouse joint interzones starting from 15.5dpc.\textsuperscript{5} With time, the expression of Prg4 increases and Gdf5 decreases.\textsuperscript{5} Prg4 expression persists throughout adult life at the surface of the articular cartilage and within the synovium but is not detectable in the growth plate cartilage.\textsuperscript{173} Prg4+ progenitor cells within the superficial layer of the articular cartilage contribute to the turnover of chondrocytes of the non-calcified cartilage layers throughout life\textsuperscript{42} but it is likely that it is the synovium-residing Prg4+ progenitors that contribute to cartilage healing after injury.\textsuperscript{37} By combining nucleoside labeling, Prg4 lineage tracking and Confetti mice, Decker et al. showed that Prg4+ progenitors proliferated within the synovial membrane, migrated and contributed to repair of cartilage defects. Prg4+ cells residing within the cartilage adjacent to the defect sites did not proliferate or contribute to repair.\textsuperscript{37} Interestingly, Seol et al. showed that the transcription factor Hmgb-1 released by dying chondrocytes functions as a chemotactant for Prg4+ progenitor cells, thereby possibly supplying a mechanistic explanation of their recruitment to the site of damage.\textsuperscript{174}

The overlap between the Gdf5+ and the Prg4+ progenitor populations in mature cartilage is not definitively established, given the absence of dual lineage tracking, however, experiments from the Pacifici laboratory suggest that Prg4 positivity is a feature of the differentiating Gdf5+ cells.\textsuperscript{5}

3.3 | Summary

The identification of different stem cell lineages with unique roles during development, homeostasis or the repair of musculoskeletal tissues is a major step forward in regenerative medicine. The understanding of the molecular control of stem cell niches, their maintenance, proliferation, migration and differentiation is revealing molecular tools to trigger repair by mobilizing progenitor cells. This may enable timely morphogenesis of the repair tissues without the need for expensive and laborious cell manipulations outside of the body. These approaches will generate upscalable, affordable production of effective and safe off-the-shelf therapeutics for the treatment of cartilage defects or osteoarthritis.
4 | CONCLUSIONS

As described in this review, several molecular targets are now available to treat cartilage degeneration. Clinical success will depend on our ability to understand the hierarchy of the homeostatic signals and the reason for repair failure in individual patients.

**Molecular hierarchy.** Interactions between the biological pathways and stem cells that contribute to skeletal homeostasis makes it hard to unpick the role of individual molecules, and the changing landscape of ligand/receptor/signaling molecule expression as cartilage degenerates must be carefully considered. The identification of key players that can initiate the repair cascade, from the recruitment of the stem cells from their niches to establishing morphogenesis of the repair tissues, without affecting the surrounding healthy tissues will be key to achieve affordable, effective and safe therapeutic interventions.

**Patient stratification.** Failure of repair/homeostasis in different patients may depend on different mechanisms. For instance, some patients may fail to repair because of poor stem cell recruitment, while others will progress because of poor tissue patterning or failure of differentiation. The selection of the right treatment for the right patient can be achieved with the identification of downstream targets of the homeostatic signals which can be used to identify the failure mechanism and at the same time as surrogate efficacy markers.

**Pharmacokinetics.** The timing, duration, delivery method and dosing of the interventions within the appropriate tissues will be critical. For instance, a brief, well-dosed activation of WNT signaling is beneficial for cartilage health under certain conditions, but persistent WNT activation is detrimental and even a burst of activation in patients with already established osteoarthritis, where WNT signaling is already over-activated, may be detrimental. Understanding the pathological processes in individual patients, combined to the availability of controlled delivery systems will be key to success.

The understanding of the developmental mechanisms of joint morphogenesis has enabled the identification of individual targets and, we predict, will continue to be crucial to address some of these key issues.

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CONFLICT OF INTEREST

Professor Dell’Accio has obtained consultancy fees from Samumed and UCB Pharma.

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