Unfolding the Mystery Behind the Onset of Chondrocyte Hypertrophy during Chondrogenesis: Toward Designing Advanced Permanent Cartilage-mimetic Biomaterials

Nilotpal Majumder and Sourabh Ghosh*

Successful recapitulation of the anatomical microarchitecture and biomechanics of the native articular cartilage under in vitro culture conditions is still an elusive topic of research. The major roadblock lies in maintaining the stable chondrogenic phenotype in vivo or under long-term in vitro conditions. Tissue engineers worldwide has coined this aberrant loss of permanent cartilage characteristics to transient cartilage form as "chondrocyte hypertrophy". Although the following has been validated through the expression of a few known markers but very little is understood regarding the molecular mechanism that dwells underneath. This review summarizes the precise aetiology behind the development and progression of the hypertrophic phenotype in chondrocytes under in vitro chondrogenic conditions. Based on the current literature survey, it is deciphered that the type of cell utilized (chondrocytes or stem cells), the chondrogenic culture conditions (growth factors/biochemical mediators) and the culture microenvironment (oxygen tension, mechanical loading) during chondrogenesis have a direct correlation with the dysregulated activity of the chondrogenic signaling pathways corroborating the onset of hypertrophic maturation of chondrocytes. Furthermore, it is critically analyzed whether to completely inhibit these hypertrophy-inducing signaling pathways or apply a brake in terms of time-dependent dose due to their functional duality role in chondrogenesis.

1. Introduction

The articular cartilage is a highly specialized connective tissue constituting articular chondrocytes as the main cell-type, 15–20% of type-II collagen (COL-II) arranged in an isotropic fashion, 4–7% of different sized proteoglycans, without any vasculature and neural tissue. The spatial arrangement of chondrocytes and collagen fibrils varies concerning the different anatomical layers of the articular cartilage. When damaged, the extracellular matrix (ECM)-trapped chondrocytes initiate repair, by clustering and producing enhanced pericellular matrix components. But a plethora of factors is responsible for the impairment in articular cartilage function that eventually leads to Osteoarthritis (OA). Aging, traumatic injury, excessive physical exercise, obesity, hyperlipidaemia, elevation in blood sugar level as well as estrogen deficiency (menopause women), synovial inflammation and osteophyte development are considered as the key risk elements that accelerate the degeneration of healthy functioning articular cartilage. For instance, during obesity, a wide variety of pro-inflammatory cytokines and adipokines are released in the adipose tissue, which has a direct contribution in orchestrating the inflammatory cascade of OA phenotype, whereas the impairment in estrogen availability in post-menopausal women interrupts the glycosaminoglycan and collagen synthesis. The clinical manifestation of OA are alarming pain and swelling in the joint region with temporary to permanent mechanical stiffness in certain cases. Clinicians worldwide are majorly focused on pain management therapy by prescribing various non-steroidal anti-inflammatory drugs (NSAIDs), visco-supplementation, as few available non-surgical therapeutic approaches to contain the symptoms of OA. The surgical treatment strategy in such cases further aggravates the condition of the patient with additional problems of post-operative pain, joint stiffness and in situ infections. This compelled the researchers to adopt tissue engineering-based strategies to develop phenotypically stable articular cartilage construct as a long-term symptomatic relief to OA manifestation by replacing the damaged tissue. Various research groups have deployed a wide array of biomaterials (silk, chitosan, collagen, alginate, PEG) and cell source (Bone marrow-derived Mesenchymal Stem Cells, Adipose-derived Stem Cells, articular chondrocytes, induced Pluripotent Stem Cells) combination to fabricate this complex tissue phenotype using exogenous addition of pro-chondrogenic growth factors like Transforming Growth Factor-β (TGF-β), Bone Morphogenetic Protein (BMP), Growth Differentiation Factor-5 (GDF-5) and Fibroblast Growth Factor -2 (FGF-2). Despite all these extensive efforts in replicating the native...
Histology of a) Healthy cartilage and b) Osteoarthritic cartilage. Dense Safranin O staining in healthy cartilage envisages the presence of enormous glycosaminoglycan content, whereas negligible positive staining is observed in the case of osteoarthritic cartilage samples. The fibrillation of the superficial layer and cellular aggregation clearly distinguishes the hypertrophic cartilage from the healthy cartilage marked by an intact superficial layer and homogenous cellular distribution.

Figure 1. Histology of a) Healthy cartilage and b) Osteoarthritic cartilage. Dense Safranin O staining in healthy cartilage envisages the presence of enormous glycosaminoglycan content, whereas negligible positive staining is observed in the case of osteoarthritic cartilage samples. The fibrillation of the superficial layer and cellular aggregation clearly distinguishes the hypertrophic cartilage from the healthy cartilage marked by an intact superficial layer and homogenous cellular distribution.

The main reason for this failure lies in the traditional cartilage tissue engineering protocol ultimately leading to an undesirable conversion of the newly formed articular cartilage to a “transient” phenotype through the process of “chondrocyte hypertrophy”, rather than re-establishment of “permanent” articular cartilage. Thus, post implantation, the cartilage construct in the defect site eventually loses its chondrocyte phenotype and initiate hypertrophic traits like vascular sprouting[26] and matrix calcification.[27] The former has been justified by the cartilage biologists as the inability of the resident cells to inhibit the activation of permanent-to-transient cartilage transformation signaling pathways, thereby reducing the efficiency of tissue-engineered cartilage in pre-clinical studies. Moreover, the differences in the differentiation of permanent articular cartilage and transient cartilage during embryonic development is not comprehensively studied or understood by the researchers. Therefore, a major research gap between identifying the challenge of maintaining a stable chondrogenic phenotype in tissue-engineered cartilage and developing OA models to screen anti-OA drugs lies in understanding the underlying mechanism governing the hypertrophic differentiation of chondrocytes. Taken together, the onset of chondrocyte hypertrophy is getting identified as a potential barrier during the clinical translation of the tissue-engineered cartilage. There are attempted strategies to attenuate the expression of hypertrophy markers, but only a handful of research has directly pointed out “how” and “why” such terminal differentiation of chondrocytes occur.

Thus, in our present review, we tried to understand the aetiology of chondrocyte hypertrophy as a routine barrier phenomenon in cartilage tissue engineering. Specifically, we aimed to raise the following questions:

1) Are all articular chondrocytes or chondrogenically differentiated MSCs destined to undergo hypertrophic differentiation following de-differentiation or embryonic development when exposed to chondrogenic stimulus in vitro?
2) Does the exogenous addition of the signaling molecules and morphogens or mimicking the biomechanical condition of native cartilage tissue for chondrogenic differentiation trigger unwanted activation of the signaling pathways leading to hypertrophy phenotype of chondrocytes?
3) Should we completely “block” or apply a “brake” to cartilage hypertrophy to achieve a stable chondrocyte phenotype?

These questions will be answered by formulating logical hypotheses based on existing literature. Understanding this rationale will enable researchers and clinicians worldwide to devise more efficient regenerative strategies to engineer articular cartilage in vitro while maintaining its stable phenotype and physiological function in vivo (Figure 1).

2. The Molecular and Cellular Level Differences in Permanent Articular Cartilage and Transient Cartilage

2.1. Articular Cartilage Development

The articular cartilage development commences with the mesenchymal condensation step, where the pre-chondrocyte mesenchymal stem cells (MSCs) increase their volume occupancy without increasing their proliferation rate with increased expression SOX9 and COL-II.[28] The cellular aggregation due to
cell-cell interaction causes deposition of different collagen matrices (COL-II, Collagen-IX, and Collagen-XI), proteoglycans (decorin, biglycan, and aggrecan) and downregulation of COL-I secretion followed by the induction of chondrogenic differentiation.\(^ {[29]}\) The interzone formation marks the initiation of continuous cartilage template segmentation. The cells present in the three-layered interzone assume a flattened morphology with a redundant expression of COL-II and SOX9 along with enhanced expression of GDF-5, Autotaxin (ATX) and Chordin in the surrounding chondrogenic layers. The interzone is also a harbour of Wnt ligands (Wnt 9a and Wnt 4) that essentially promotes the expression of articular cartilage specific genes (GDF5, ATX, and Chordin) in the interzone cells.\(^ {[30,31]}\) The permanent articular cartilage differentiation originates from the cells near proximity of the interzone. A wide constellation of transcription factors aid in the chondrogenic differentiation of the mesenchymal cells among which C-1-1 and OSR1/2 plays a significant role in stimulating Wnt ligand secretion and further blocking transient cartilage differentiation.\(^ {[32,33]}\) A transcription factor c-Jun is also known to promote articular cartilage differentiation by promoting the transcription of Wnt 9a and Wnt 4 ligands.\(^ {[34]}\) There are also evidences of presence of GATA3 gene in the articular cartilage tissue that significantly regulated the expression of permanent cartilage specific genes (ATX and SERP2)\(^ {[35,30]}\)

### 2.2. Transient Cartilage Development

The transient cartilage differentiation, unlike the permanent articular cartilage, originates from the center of the cartilage template constituting a pool of COL-II expressing cells located at a far distance from the region of permanent articular cartilage differentiation. The latter being the major source of Parathyroid Hormone Related Peptide (PTHrP) that inhibits transient cartilage differentiation. During the growth of the cartilage template (also known as cartilage analog), the cells evade the influence of PTHrP signaling cascade and immediately activate Indian Hedgehog (IHH) pathway initiating the hypertrophic differentiation process.\(^ {[36,37,38]}\) The involvement of BMP signaling pathway in endochondral ossification\(^ {[39]}\) is well explained by Biswas et al. where the BMP induced activation of ROS scavenging enzyme Prdx1 significantly upregulated the IHH expression in the differentiation chondrocytes.\(^ {[40]}\) Furthermore, the expression of osteoblast actin assembly gene Dspy3 in the hypertrophic chondrocytes is activated by the BMP signaling pathway.\(^ {[41]}\) The matrix remodeling and vascular sprouting regulated by the matrix metalloproteases\(^ {[42]}\) and Vascular Endothelial Growth Factors (VEGF) respectively is immediately followed by osteogenic differentiation with a remarkable increase in the expression of Runx-related transcription factor-2 (RUNX2).\(^ {[30]}\) Thus, the transient cartilage with an elevated level of Collagen-X (COL-X), Collagen-I (COL-I), and RUNX2 expression attains a bony tissue-like phenotype hugely different from permanent hyaline cartilage.

### 2.3. Common Point of Origin for Permanent and Transient Cartilage

Evidence from the existing literatures propose that permanent articular cartilage and transient cartilage originates from two distinct precursor cell populations. However, when the researchers have deployed a combination of EdU/Brdu pulse-chase analysis and COL-II mRNA lineage tracing of the proliferating chondrocyte, they successfully demonstrated that both the articular cartilage and transient cartilage are developed from a shared pool of actively proliferating cells present in the cartilage analog.\(^ {[42]}\) There is further evidence that the cells originating from the interzone contribute to the formation of transient cartilage, which demarcates formation of independent cartilage element. The cells differentiates into the respective cartilage lineage depending on the zone-specific ectopic activation of BMP and Wnt signaling pathways, respectively.\(^ {[43]}\) The cells that are restricted within the region of Wnt signaling are dedicated toward permanent articular cartilage fate insulated by a Noggin barrier,\(^ {[42]}\) whereas the cells present within the zone of BMP signaling proceed toward transient cartilage differentiation. The interzonal cells at the periphery transform to chondrocyte lineage with an increase in proteoglycan deposition.\(^ {[44]}\) Thus, interzone formation by tissue engineering should be considered as a major intermediate milestone for permanent articular cartilage segmentation (Figure 2).

### 3. Are we Close Enough to Mimic the Native Articular Cartilage Differentiation Process in vitro?

Researchers globally have explored a wide array of 2D and 3D-based approaches using vivid combinations of cell types, biomaterials, and biochemical mediators to simulate the tissue architecture, mechanical rigidity and physiological function of the native articular cartilage. When compared, the 3D culture model of articular chondrocytes revealed an advantageous role in enhanced chondrogenic biomarker expression and ECM content deposition than the 2D model.\(^ {[45]}\) Although these 3D culture systems have demonstrated chondrogenic differentiation, their ambivalent character has been equally exposed through the stable expression of terminal differentiation biomarkers like COL-I and COL-X. Thus, the in vitro chondrogenic stimulus to the BMSCs and primary chondrocytes led to the formation of mechanically inferior “fibrocartilage” instead of “hyaline cartilage”, more prominently in monolayer than 3D culture conditions. This led the progressive group of tissue engineers to completely shift their paradigm to alternative 3D culture systems with a hope to achieve stable cartilage phenotype.

#### 3.1. 3D Aggregate-based or Scaffold-based Models

##### 3.1.1. 3D Aggregate

A 3D scaffold-free approach in the form of spheroid or pellet culture seemed feasible for the investigators to alleviate the onset of hypertrophy. In a dynamic culture system with ADSCs, Yoon et al. and Tu et al. demonstrated impressive chondrogenic traits in spheroids independent of the presence of TGF-\(\beta\).\(^ {[46,47]}\) A comparison between the ADSC spheroids and chondrocyte spheroid also revealed a higher level of ECM deposition in ADSC spheroids than in chondrocytes, while the expression profile of COL-II followed a reverse order.\(^ {[48]}\) Such acc characteristics of the spheroid culture system has additional advantage of increased...
cell-cell communication and establishing a hypoxic microenvironment during development of articular cartilage tissue.\(^{[49]}\) However, Zhang et al. underlined the overall advantage of micro-mass culture over pellet culture using BMSCs in achieving articular cartilage specific COL-II expression with diminished fibro-cartilage marker expressions.\(^{[50]}\) Researchers have also reported the use of minimally expanded human articular chondrocytes in developing spheroid-based microcartilage models depicting enhanced secretion of COL-II and glycosaminoglycans (GAG) along with a sturdy expression of hypertrophic marker COL-X.\(^{[51]}\) However, challenges about forming the necrotic core at the center of the spheroid, non-uniformity in ECM production and the stunted proliferation rate of the involved cells led tissue engineers to contrive an alternative method to induce a stable phenotype in long-term culture conditions.

3.1.2. 3D Scaffold-based

When primary chondrocytes, human BMSCs, human ADSCs, ESC (H9, WiCell Research Institute), and ESC-derived mesenchymal stem cells (developed by two different protocols, ED1 and ED2) were differentiated in silk and chitosan scaffolds with and without BMP-6 in chondrogenic media, BMP-6-modified silk scaffolds containing ED1 MSCs demonstrated the best potential for cartilage regeneration. However, both the ED1 and ED2 cells in the scaffolds depicted maximum hypertrophic features.\(^{[52]}\) The challenge of using primary chondrocytes is that only the last-stage human chondrocytes is accessible for research purposes extracted during the cartilage reconstructive surgery. Such OA articular chondrocytes displayed an altered phenotype compared to normal chondrocytes, akin to growth plate chondrocytes. Thus, findings from their research clearly demonstrated the onset of hypertrophic maturation in the chondrocytes.\(^{[53]}\) Therefore, adopting a 3D culture system (scaffold-based and scaffold-free) could not yet solve the problem with chondrocyte hypertrophy and achieve stable 3D cartilage tissue.

3.2. 3D Bioprinted Models

3.2.1. 3D Bioprinted Dispersed Cells

Earlier research from our laboratory has extensively elucidated the role of silk-based biomaterial and 3D bioprinting strategies in instructing chondrogenic differentiation of BMSCs and articular chondrocytes while firmly resisting the induction of hypertrophy. Chameettachal et al.\(^{[14]}\) utilized the same SF-G bioink in developing a cartilaginous tissue construct using BMSCs and articular chondrocytes in a 3D system. The results depicted the advantage of using primary chondrocytes over BMSCs in 3D bioprinted constructs in mitigating chondrocyte hypertrophy while promoting chondrogenesis. The advantages of spatio-temporal control on the deposition of the desired bioink and controlled porosity resulted due to 3D bioprinting, along with the chondrogenic role of silk fibroin biomaterial, have been exploited by Chawla et al.\(^{[19]}\) They used a combination of silk fibroin-gelatin (SF-G) blend and BMSCs to fabricate a tissue-engineered cartilaginous construct and assessed the additional role of growth factor TGF-\(\beta\)1 in chondrogenesis. Their investigation demonstrated the upper hand of using a combination of silk bioink-based 3D bioprinting technique in developing cartilage constructs owing to their advantage in activating pro-chondrogenic Wnt/\(\beta\)-catenin signaling cascade and spatio-temporal control over cell encapsulated biomaterial deposition and morphogen bioavailability.\(^{[19,54]}\) Investigations have been carried out by several other researchers using 3D bioprinting on a similar line, for instance, using a combination of nanocellulose and alginate embedded with human chondrocytes (Markstedt et al.),\(^{[55]}\) collagen hydrogel and human naso-septal chondrocytes (Lan et al.),\(^{[56]}\) and MSCs encapsulated in norbornene conjugated hyaluronic acid hydrogel for in situ photo-crosslinking (Galarraga et al.).\(^{[57]}\) Despite such tremendous efforts, the fabricated tissue constructs fail to maintain the chondrogenic property when cultured for a longer period under chondrogenic stimulus.
3.2.2. 3D Bioprinted Spheroids

To overcome this challenge on chondrocyte hypertrophy, tissue engineers have adopted an advanced strategy of directly 3D bio-printing the spheroids. This method was rightly adopted by Melo et al., employing fibrin encapsulated BMSC spheroids bioprinted over a PEG-alginate bath. The spheroids successfully maintained the chondrogenic phenotype without interfering with the mechanical properties of the developed hydrogel system.\cite{Melo2018} Moor et al. also demonstrated the superior chondrogenic characteristics (COL-II and GAG secretion) of 3D bioprinted BMSC spheroid encapsulated gelatin methacrylate (GelMA) constructs as compared to dispersed cells in the hydrogel. The authors assumed the mechanical robustness of the already secreted ECM of the spheroids in vitro to be the primary reason behind maintaining such increased cell viability and chondrogenicity while withstanding high shear stress during 3D printing.\cite{Moor2018} On a similar note, Nguyen et al.\cite{Nguyen2018} and Scalzone et al.\cite{Scalzone2018} used a coculture system of induced pluripotent stem cells (iPSCs) as well as BMSCs with chondrocytes (spheroid and dispersed) encapsulated in respective alginate and chitosan-based bioink to develop a 3D bioprinted in vitro model of articular cartilage. However, results from all these attempts have clearly indicated the simultaneous hypertrophic maturation of the developed neo-cartilage, even utilizing the most advanced technique of tissue engineering.

Nonetheless, the efforts put forward by the cartilage tissue engineers in collaboration with clinicians and cell biologists led to the formation of engineered cartilage tissue in vitro, the onset of further transdifferentiation could not be avoided in every single culture system adopted (monolayer, 3D spheroids, and 3D bioprinted). The reason for such hypertrophic conjecture of BMSCs or primary chondrocytes during chondrogenic differentiation in vitro is still a riddle to be resolved. Therefore, there exists a paradoxical relationship where exogenous addition of the pro-chondrogenic cytokines or small molecule inhibitors of bone-forming signaling cascades in chondrogenic differentiation medium, although significantly induced chondrogenesis of the cultured mesenchymal stem cells or primary chondrocytes but did not attenuate their transient cartilage differentiation.\cite{Nguyen2018, Scalzone2018} Thus, it can be postulated that under the influence of chondrogenic stimulus in vitro, the MSCs and chondrocytes (monolayer, 3D spheroid or 3D scaffold) demonstrate an intermediate unstable chondrogenic phenotype that eventually undergoes hypertrophic differentiation to finally transform into a transient cartilage. Henceforth, the current available cartilage tissue engineering strategies have expedited the development of an inconsistent or “pseudo-chondrogenic” status of newly formed chondrogenic tissue during in vitro chondrogenic differentiation.

4. A Dire Need to Refine the Molecular Characterization of the Tissue-Engineered Cartilage

The biomarkers that characterize a tissue-engineered cartilage can be broadly categorized as a “clinical phenotype” and an “endotype”. When various observable attributes like the causative agents and their associated risk factors distinguish a particular subpopulation of a cell (chondrocytes) in a tissue, it is termed as a clinical phenotype.\cite{Lieber2018} However, when the underlying cellular and molecular events in disease are considered in characterizing the specific tissue-type, such as the presence of various downstream signaling targets governing the ectopic activation of respective cellular signaling cascades, it is termed an endotype.\cite{Greco2020, Lieber2018} The expression of a single hypertrophic marker can be observed in different degrees governed by different underlying pathways signifying different stages of disease progression. Furthermore, due to the multi-layer anatomy of articular cartilage (Figure 1), the gene expression patterns might also vary with different layers based on their biochemical and molecular composition. Grogan et al. have extensively studied these gene expression profiles at different zones of articular cartilage.\cite{Grogan2018} Their research indeed reveals the varied expression of several markers like aggregan (ACAN) and COL-II, exhibiting minimum expression in the superficial zone but extensively present in the mid-zone.\cite{Grogan2018} Several other scientists have also deciphered different biomarkers at both clinical and molecular levels governing the formation of tissue-engineered cartilage in vitro from a wide range of cell sources.\cite{Greco2020, Lieber2018} Therefore, there is a dire need to refine the protocol for molecular characterization of the tissue-engineered cartilage to compare with the articular cartilage tissue formed during embryonic development in vivo.

4.1. Commonly Analyzed Biomarkers

The tissue-engineered cartilage developed using various scaffold-based, or scaffold-free approaches are validated through mRNA expressions of specific biomarkers. Among them, COL-II, SOX-9, and ACAN are universally used in evaluating the development of hyaline articular cartilage. SOX9 protein during neo-cartilage formation is known to aid in the maintenance of cell morphology required during the mesenchymal condensation stage, besides regulating the expression of other pro-chondrogenic markers like ACAN and COL-II. The deposition of proteoglycans and collagen matrices during the chondrogenic differentiation phase of embryonic development is marked by the expression of ACAN and COL-II, whereas the hypertrophic differentiation of the developed chondrocyte phenotype is generally confirmed by the secretion of mechanically inferior collagen matrices (COL-X and COL-I) as well as the matrix metalloproteases (MMP-1/13). The expressions of these biomarkers have been extensively quantified using RT-PCR by various research groups as the only benchmarks for successful chondrogenesis.\cite{Greco2020, Lieber2018} However, one cannot fully validate successful chondrogenic differentiation without examining several associated biomarkers that has a significant role in the physiological functioning of the articular cartilage.

4.2. Specialized Biomarkers

Proteoglycan 4 (PRG4) or lubricin is a superficial zone-based marker of articular cartilage (also known as superficial zone protein) composed of mucin-rich glycoproteins that aids in the friction-free movement of the gliding joints by providing smooth lubrication. At the same time, it arrests the progression of OA in the developing cartilage by limiting the synoviocytes proliferation. The chondroprotective role of PRG4 has been listed in
several in vitro and in vivo investigations by Chawla et al.\textsuperscript{[19]} Alquaraini et al.\textsuperscript{[70]} and Lefebvre et al.\textsuperscript{[71]} correlated with the expression profiles of other pro-chondrogenic genes. Another definitive articular cartilage differentiation marker is Autotaxin (ATX) present in chondrocyte’s plasma membrane that regulates the production of lysophosphatidic acid (LPA), which in turn is a potent regulator of fibrocartilage formation through COL-I expression.\textsuperscript{[72,73]} The downregulation of such ATX expression post 21 days culture of BMSCs in chondrogenic media has been reported by Chawla et al., necessitating the chondro-regulatory role of ATX in articular cartilage development.\textsuperscript{[19]} Additionally, Singh et al have reported two new transcription factors, NFIA and GATA3, as a confirmary marker for articular cartilage differentiation. The study revealed the hypertrophy inhibiting role of NFIA and the chondro-stimulating role of GATA3 in articular cartilage tissue.\textsuperscript{[14]} Hence, evaluating the potential mRNA expressions of PRG4, ATX, NFIA, GATA3, etc in the newly fabricated cartilage tissue is an appropriate strategy to authenticate the complete recapitulation of anatomy and physiology of the native articular cartilage tissue.

### 4.3. Signaling Pathway-Specific Biomarkers

Any embryonic tissue development process is regulated by a stringent mechanistic crosstalk between several cellular signaling pathways and the positive and negative expressions of their respective downstream targets that controls the fate of the neotissue. However, this portion is generally overlooked owing to its complex unsolved molecular level understandings. Our group has been instrumental in deciphering the regulatory role of chondrogenic signaling pathway downstream targets (β-catenin, SMAD4) with enhanced expression of β-catenin and catastrophic decrease in SMAD4 expression during chondrogenic differentiation of BMSCs and primary chondrocytes respectively.\textsuperscript{[14,19]} Moreover, we are the first group to unfold the pro-Wnt characteristics of silk fibroin biomembrane in stimulating in vitro chondrogenesis by enhanced expression of β-catenin, as assessed from detailed proteomic analysis.\textsuperscript{[74]} Silk-gelatin bioink also upregulated several anabolic chondrogenic signaling pathways (Notch, integrin signaling pathways, HIF-1) and inhibited catabolic pathways such as NF-κB signaling. Hypoxia through its transcription factors HIFα and Histone deacetylase (HDAC4), has been a crucial regulator of vascular invasion and sprouting angiogenesis during the hypertrophic matura-

### 5. The Mystery Behind the Futile Cartilage Tissue Engineering Strategies: Chondrocyte Hypertrophy

The terminally differentiated chondrocytes display an increase in cell volume due to osmotic swelling.\textsuperscript{[77]} Such change in osmolarity is attributed to the decrease in extracellular concentration or increase in cytoplasmic concentration, increase in cellular organelle and ECM degradation around the periphery of the cell.\textsuperscript{[78]} The alteration in osmolarity causes intracellular movement of water through aquaporins and aid in bone tissue formation.\textsuperscript{[79]} However, it is still poorly understood whether the increase in cellular volume a biomarker for terminal chondrocyte differentia-

### 6. Unfolding the Mystery: The Underlying Mechanisms Governing Chondrocyte Hypertrophy

Numerous research has been carried out to decipher the molecular symptoms and the associated regulators of hypertrophy but the etiology and the underlying mechanism governing the process still remains an unsolved puzzle. To unfold this mystery, we have postulated the following hypotheses (Summarized in Table 1) to understand and elucidate the anticipated reasons behind the occurrence of this pleiotropic phenomenon.
<table>
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<th>Serial no.</th>
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<th>Key observations</th>
<th>Probable underlying mechanism</th>
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<tr>
<td>1.</td>
<td>Dedifferentiation of primary chondrocytes during in vitro expansion protocol</td>
<td>Stressed actin fibres with vimentin pawning over the cytoplasm in 2D chondrocyte culture system&lt;br&gt;Enhanced expression of α-SMA in higher passage chondrocytes resulting in biomechanically inferior tissue&lt;br&gt;Downregulated expression of chondrogenic marker genes (COL-II, COMP-1, SOX9, ACAN, chondromodulin).&lt;br&gt;Stimulated the secretion of COL-I with elevated expression of hypertrophy markers ALP, RUNX2, COL-X</td>
<td>Actin polymerization and presence of nuclear myocardin related transcription factor a (MRTFa) in de-differentiated chondrocytes.&lt;br&gt;Enhanced expression of 𝛼-SMA in higher passage chondrocytes resulting in biomechanically inferior tissue&lt;br&gt;Downregulated expression of chondrogenic marker genes (COL-II, COMP-1, SOX9, ACAN, chondromodulin).&lt;br&gt;Stimulated the secretion of COL-I with elevated expression of hypertrophy markers ALP, RUNX2, COL-X</td>
<td>[173,174] [98,99,100,101,102]</td>
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<td>2.</td>
<td>The natural tendency of stem cells to undergo hypertrophic maturation: dysregulation in the chondrogenic pathways</td>
<td>A 21 days TGF-β-1 exposure to BMSCs demonstrated enhanced expression of hypertrophic markers COL-X and IHH.&lt;br&gt;Muscle limb MSCs when exposed to TGF-β-1 caused enlarged cell morphology, extensive mineralization followed by abundant expression of VEGF, COL-X and Osteocalcin post 21 days of culture.&lt;br&gt;Media supplementation with BMP-2 protein during the chondrogenic differentiation of MSCs and ADSCs displayed prominent hypertrophic traits marked by elevated expression of COL-X, COL-I, ALP, IHH, RUNX2 and Osteopontin.&lt;br&gt;Wnt proteins (Wnt 3a, 8c and 9c) and agonists (Melatonin and BIO) demonstrated significant upregulation of hypertrophic maturation gene expressions (COL-X, RUNX2, ALP, ADAMTS5) along with enhanced matrix calcification.&lt;br&gt;Dexamethasone addition to the chondrogenic differentiation medium caused significant increase in ALP activity and suppressed proteoglycan synthesis and accumulation in the surrounding ECM.&lt;br&gt;Exogenous treatment of BMSCs with Platelet rich plasma during chondrogenesis elevated the expression of COL-I and Osteocalcin</td>
<td>The variation in concentration and time of exposure of TGF-β-1 during chondrogenic differentiation of stem cells.&lt;br&gt;The initiation of Smad 1/5/8 pathway through ALK-1 activation by TGFβ1 and non-canonical MAPK pathway&lt;br&gt;The difference in time-point of addition of BMP protein during chondrogenesis, the variant of BMP (BMP-2/4/6/7/9) used as a media supplement or the concentration and exposure time.&lt;br&gt;The aberrant crosstalk between TGFβ1 and BMP signaling pathway followed by dysregulated activation of RUNX2 transcription via Smad-1/5/8 canonical pathway&lt;br&gt;The ligand mediated activation of hypertrophic canonical Wnt signaling cascade or the intracellular signaling crosstalk between several hypertrophy inducing signaling pathways&lt;br&gt;A disbalance in the exposure time and concentration during in vitro chondrogenic differentiation of stem cells.</td>
<td>[19,175,109,110,112] [176,115,177,117,118] [119,124,123,178] [179,180] [20,181]</td>
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<tr>
<td>1.</td>
<td>IHH signaling pathway independently or in association with several other osteogenic signaling cascade (BMP) depicted elevated hypertrophic traits marked by overexpression of matrix degrading enzymes (MMP-13) and COL-X.</td>
<td>Through the post-translational modification of secreted collagen during chondrogenesis.</td>
<td>The innate role of IHH</td>
<td>[76,134]</td>
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<td>2.</td>
<td>The ubiquitous role of hypoxia during chondrogenic differentiation</td>
<td>HIF-1α promoted the autophagy of chondrocytes by suppressing the activity of Bcl-2 and caspase-8 protein, a key trait observed in hypertrophic chondrocytes.</td>
<td>The activation of pro-inflammatory NF-κB pathway causing ECM degradation.</td>
<td>[137,138]</td>
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<td>3.</td>
<td>HIF-2α is a potent transactivator of several endochondral ossification pathway genes like COL-10, MMP-13, and VEGF. The expression of HIF-2α gene is highly promoted by the presence of hydrostatic pressure due to the higher proportion of water content in the cartilage tissue.</td>
<td>The transcription of pro-hypertrophic marker genes through the activation of C/EBPβ pathway. Hydrostatic pressure promotes cartilage matrix degeneration through HIF-2α mediated secretion of Aggrecanase and matrix degrading proteases along with HIF-2α independent activation of NF-κB pathway.</td>
<td>[140,141]</td>
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<td>4.</td>
<td>The imbalance in mechanical loading during in vitro chondrogenesis</td>
<td>Excessive mechanical compression, oscillatory hydrostatic pressure, shear force and tensile loading enhanced the expression of catabolic matrix degrading enzymes (MMP-13 and ADAMTS5) along with suppression of pro-chondrogenic gene expressions.</td>
<td>Activation of TNF-α, IL-1β, NF-κB, Wnt and TGF-β and their mechanistic crosstalk</td>
<td>[142,143]</td>
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Figure 3. H&E staining of healthy and hypertrophic cartilage demonstrates increased cell volume and cell clustering in the hypertrophic cartilage. The possible reason can be attributed to the osmotic influx of water through the aquaporin channels due to the change in osmolarity of the hypertrophic microenvironment. A pertinent question is whether the increase in chondrocyte volume and clustering arise due to hypertrophic maturation or whether this chondrocyte swelling is associated with abnormal (neo)matrix production and directly/indirectly causes cartilage graft failure. Moreover, when the isolated chondrocytes are expanded in vitro in 2D, they lose their round morphology and assume a fibroblastic characteristic marked with an increased expression of COL-I instead of COL-II through a process termed ‘cellular de-differentiation’. When these cells are cultured in 3D systems (embedded in a hydrogel), the round morphology of the chondrocyte is restored with an expression of articular cartilage-specific markers. However, the ambiguity lies in the fact that if the “cellular de-differentiation” phenomenon can be reversed in 3D culture models, what could be the possible source of chondrocyte hypertrophy when the same chondrocyte-laden tissue constructs are implanted in vivo?

6.1. The tendency of the Articular Chondrocytes to Undergo de-differentiation During Chondrogenesis

For tissue engineering-based approaches, obtaining a high cell density is a critical variable to ensure homogenous secretion of ECM component and expression of enough cell-surface receptors to activate downstream signaling cascades. During the monolayer expansion of chondrocytes (irrespective of the origin), they inevitably loose their native cellular morphology along with their physiological function. The expansion phase of chondrocytes results in a more fibroblastic morphology of chondrocytes instead of a round one with a significant increase in cell volume. The critical interplay between the altered morphology of chondrocytes and the alteration of cytoskeleton assembly with increased expression of focal adhesion complex and thick F-actin fibres have been duly reported as the molecular changes underneath. A significant downregulation of COL-II secretion and aggrecan is marked by an equivocal increase in COL-I secretion and small sized proteoglycans (decorin and biglycan) thereby altering the overall biomechanical property of the surrounding matrix. The expression of several other pro-chondrogenic markers like SOX9, chondromodulin, COMP-1 and superficial zone protein lubricin also diminished during the proliferative stage of chondrocytes. There are reports that also showed the elevated expression of several transient cartilage biomarkers like ALP and RUNX2 during the monolayer expansion of chondrocytes.

Researchers have tried to investigate the underlying mechanism behind the occurrence of such unusual dedifferentiation phenomenon and concluded the involvement of several cell signaling pathways like Mitogen-activated Protein Kinase (MAPK), p38, Extracellular Signal-regulated Kinase (ERK), and phosphoinositide 3-kinase (PI3K) governing the entire process of chondrocyte dedifferentiation. As already discussed in section 3.2 that an increase in cellular volume followed by a catastrophic decrease in chondrogenic biomarkers (COL-II, SOX9, and ACAN) with an increased expression of bone-related markers (RUNX2 and Alkaline Phosphatase) are the key phenotypic alterations in a hypertrophic chondrocyte. Thus, it can be rightly postulated that the acquired dedifferentiated phenotype of primary articular chondrocytes during chondrogenic differentiation can be attributed to one of the possible reasons behind the hypertrophic maturation of the cultured chondrocytes.
6.2. The natural Propensity of Stem Cells to Attain Transient Phenotype under the Influence of Different Chondrogenic Media Supplementation: Dysregulated Activation of Chondrogenic Signaling Pathways

The stem cells possess excellent proliferation and differentiation capability unlike autologous chondrocytes making them a perfect fit for tissue engineering applications. The BMSCs and ADSCs have been widely explored by the tissue engineers both in pellet form as well as embedded in the 3D scaffolds for chondrogenesis due to their easy isolation procedures besides bearing the general properties of other stem cells. BMSCs cultured in 3D bioprinted SF-G construct under the continuous chondrogenic stimulus for 28 days demonstrated excellent chondrogenic potential whereas the same chondrogenic stimulus for 21 days caused the resurgence of hypertrophic traits. Similarly, ADSCs are one such cell source that is widely isolated from the infrapatellar pad (IFP) or abdomen tissue that intrinsically express SOX-9 and exhibit higher proliferation rate than BMSCs. However, these cells also express osteogenic differentiation marker RUNX2 marking their inclination toward hypertrophic maturation. When compared with BMSCs under the influence of chondrogenic morphogens, ADSCs displayed an increased tendency toward hypertrophic maturation with elevated levels of COL-X and COL-I expression but the tendency was reported to be less than BMSCs. The commonly used differentiating agents for the chondrogenic differentiation of stem cells are ascorbic acid, insulin, TGF-β1/3, Wnt ligands and BMP proteins. The exogenous addition of these chondrogenic signaling agents during in vitro chondrogenesis often activates certain transient cartilage-forming signaling cascades resulting in the transforming the permanent cartilage phenotype to a hypertrophic state.

6.2.1. Transforming Growth Factor Beta Isoform (TGF-β)

The TGF-β superfamily morphogens exhibits a functional duality role during chondrogenesis by activating both chondrogenic and hypertrophic differentiation route. The Smad-dependent route or the canonical pathway of TGF-β mainly operates via the ALK-5 phosphorylation-mediated activation of their downstream Smad targets (Smad2/3) forming a ternary complex with Smad4 and inhibit the hypertrophic differentiation of chondrocytes. However, there are reports that suggests that TGF-β can also function via the phosphorylation of ALK-1 receptor activating Smad1/5/8 signaling pathway independent of ALK-5 activation. The RUNX2-Smad1 complex formed, highly elevates the function of the RUNX2 protein thereby promoting the hypertrophic maturation of the chondrocytes toward an osteogenic lineage. The TGF-β also activates the hypertrophic marker expression, RUNX2 in mesenchymal cells by initiating the non-canonical MAPKs signaling pathway with JunB as the upstream signaling target. These findings clearly augment the hypothesis that chondrogenically differentiated stem cells have been observed. Thus, the unwanted activation of Smad1/5/8 and MAPK dependent TGF-β signaling cascade can be hypothesized to be one of the key reasons for the hypertrophy inducing role of TGF-β.

6.2.2. Bone Morphogenetic Proteins (BMP)

Exogenous addition of BMP molecule to the chondrogenic differentiation media of stem cells have been reported by numerous researchers to stimulate the collagen and proteoglycan synthesis during chondrogenesis by activating the BMP signaling cascade. However, akin TGF-β cascade, BMP also functions via the canonical Smad 1/5/8 phosphorylation route activating the transcription of RUNX2 responsive hypertrophic genes ALP, COL-X, and MMP-13 confirming the functional duality of BMPs in chondrogenesis. A similar role of BMP-2 in inducing chondrocyte hypertrophy have been explained by Chawla et al. where the minimally expanded human articular chondrocytes when treated with BMP-2 molecule significantly upregulated the COL-X expression along with consistent GAG and COL-II secretion. Furthermore, both in vivo and in vitro investigations have established the mechanistic crosstalk between the TGF-β and BMP signaling cascade through the expression of each other’s respective downstream targets that regulate the hypertrophic state of chondrocytes. Therefore, we can presume that the exogenous addition of BMP molecule (dose, exposure time and time of addition) as a pro-chondrogenic factor during chondrogenesis caused an aberrant dominance of hypertrophic trait over the chondrogenic phenotype thereby promoting endochondral ossification over permanent cartilage differentiation.

6.2.3. Wingless Protein (Wnt ligands)

Researchers have examined the differential significance of various Wnt superfamily members like Wnt 3a, Wnt 4, Wnt 5a, Wnt 9a in endorsing the different stages of cartilage development and further delaying chondrocyte hypertrophy. However, the Wnt signaling pathway exhibits an ambiguous role by simultaneously promoting chondrogenesis as well as hypertrophic differentiation of chondrocytes. This was confirmed by Huang et al. in their detailed investigation by using Wnt signaling inducer (BIO) and inhibitor (PKF-118-310) as a media supplement in the chondrogenic differentiation media of MSCs. Results from their study revealed a catastrophic downregulation of chondrogenic biomarker expressions with an increase in Aggrecanase (ADAMTS5) production in the Wnt-activator group whereas enhanced levels of cartilage-specific gene expression was observed when the signaling pathway was inhibited by PKF118-310. A similar line of results have been demonstrated by several other research groups using different variants of Wnt proteins (over expression of Wnt8c and Wnt9c in chondrocytes or Wnt3a sequestration in human cartilage) and Wnt activators (Melatonin) underlying the ambivalent role of Wnt pathway in chondrogenesis. Therefore, we can suspect that the pro-chondrogenic Wnt signaling pathway under chondrogenic stimulus may transition to its hypertrophy-inducing role either due to their dysregulated ligand-mediated activation mechanism or as a part of the mechanistic crosstalk with several other hypertrophic factors.
maturation signaling cascades (BMP and IHH) as evident from the existing reports.\textsuperscript{127, 128}

6.2.4. Other Chondrogenic Mediators and Activated Hypertrophic Signaling Cascades

Insulin and ascorbic acid are two vital media supplements during in vitro chondrogenic differentiation assisting in chondrocyte proliferation and collagen synthesis respectively.\textsuperscript{129, 130} However, both these biochemical mediators induce profound hypertrophic differentiation following the endochondral ossification route following the activation of ERK 1/2 mitogenic signal transduction pathway.\textsuperscript{129, 131} Enhancing the transcription process of the downstream effectors of ERK signaling cascade (COL-X, RUNX2, ALP, and MMP-13) makes these signaling molecules a positive regulator of chondrocyte hypertrophy.\textsuperscript{128} Platelet rich plasma (PRP), a commonly used chondrogenic inducer of BMSCs also demonstrated an upregulated expression of hypertrophy marker COL-I.\textsuperscript{132} A strong correlation between BMP and IHH signaling targets has been elucidated by Minina et al. where the expression levels of IHH has been tightly controlled by BMP protein suggesting a synergistic relationship in stimulating hypertrophic hypertrophy.\textsuperscript{133} An increased expression of IHH in OA chondrocytes has been associated with enhanced expression level of hypertrophic biomarkers (MMP-13, COL-X) as elucidated by Wei et al.\textsuperscript{134}

The embryonic articular cartilage development is ultimately followed by an endochondral ossification route to finally undergo hypertrophic differentiation, a distinct strategy of bone development besides via intramembranous ossification. Thus, during in vitro chondrogenic differentiation under the influence of the pro-chondrogenic signaling morphogens, the stem cells display a pro-hypertrophic phenotype despite adequate chondrogenesis demonstrating their innate tendency following the embryonic cartilage development pathway.\textsuperscript{135} Furthermore, we can postulate that under the influence of chondrogenic media supplements during the entire period of in vitro chondrogenesis, stem cells display an ectopic dysfunctioning of several chondrogenic signal transduction pathways. Such dysregulation can be attributed to the time of exposure and concentration of the biochemical mediators deployed during the chondrogenic differentiation phase. These aberrant signaling cascades enable the permanent cartilage to shed off its native stable phenotype and acquire an osteogenic phenotype, an unanticipated outcome of chondrogenic differentiation (Figure 4).

6.3. The Ambiguous Role of Hypoxia Generated Factors

Low oxygen tension or hypoxia is considered as an ideal culture condition for chondrogenic differentiation in vitro. However, studies have also addressed the cynical role of hypoxia during chondrogenesis with an upregulated expression of crucial hypertrophic marker COL-X when BMSCs were cultured in Hyaluronic acid and collagen scaffolds.\textsuperscript{136} HIF-1\textalpha which is chondroprotective in nature also reported to promote autophagy and chondrocyte apoptosis by regulating the functioning of autophagy and apoptosis-related proteins Bcl-2 and Caspase-8.\textsuperscript{137}

A possible mechanism for this pro-apoptotic role of HIF-1\textalpha can be through the post translational modification of the secreted collagen by the differentiating chondrocytes as elucidated by Yao et al.\textsuperscript{138} Hypoxia also stimulated the production of IL-1\beta thereby creating an inflammatory niche further aggravating the condition of the already degenerated cartilage matrix during hypertrophy.\textsuperscript{139}

HIF-2\alpha, a family member of hypoxia-related proteins has prominently depicted the pro-hypertrophic role during in vitro chondrogenesis by escalating the transcription of COL-X, MMP-13, and VEGF.\textsuperscript{140} The in vivo analysis by Yang et al. also corroborated the deleterious activity of HIF-2a in promoting transient cartilage differentiation when administered via adenovirus or overexpression of HIF-2a gene.\textsuperscript{141} Moreover, a fascinating observation by Inoue et al. demonstrated that the hydrostatic pressure enhanced the expression of HIF-2a gene promoting cartilage degeneration, an eminent marker of chondrocyte hypertrophy.\textsuperscript{142} The presence of such hydrostatic pressure corresponds to high water content (~70%) in articular cartilage tissue that acts as a negative stimulus to chondrocytes causing further matrix degradation and apoptosis.\textsuperscript{143} Scientists have also described the derogatory function of hypoxia in chondrocyte hypertrophy by accelerating the production of pro-inflammatory molecules prostaglandin E-2 and cyclooxygenase-2, creating a suitable niche for OA development in the hypertrophic chondrocytes. Thus, we can presume that the so-called ‘ideal hypoxic environment for chondrogenesis can be manoeuvred during chondrogenic differentiation leading to an undesirable hypertrophic phenotype. Such manipulation can be either due to the endogenous switch from pro-chondrogenic HIF-1\textalpha to pro-hypertrophic HIF-2\alpha under hypoxic condition or are a result of the crosstalk between different dysregulated activation/suppression of chondrogenic signaling molecules (described in the next section) supplemented in media in vitro. Furthermore, HIF-1\textalpha and HIF-2\alpha promotes the process of neo-vascularization or vascular spraying in the cartilage matrix from the underlying subchondral bone, a hallmark of transdifferentiated chondrocytes. Thus, the proangiogenic property of these hypoxia-induced transcription factors equally contributes to the hypertrophic maturation of the chondrocytes when cultured in vitro (Figure 4).

6.4. The Imbalanced Mechanical Loading During in vitro Chondrogenesis

Native articular cartilage of the knee joint is exposed to a constant mechanical loading that plays a vital role in the physiological homeostasis of the tissue. This mechanical loading comprises of shear forces (parallel to the articulating surface), compression forces (constant human locomotion) and hydrostatic pressure (synovial fluid) that renders the mechano-active nature of the cartilage tissue. Thus, mimicking the native biomechanical condition during in vitro articular cartilage development is essential to establish a stable hyaline cartilage phenotype. Investigation by Nazempour et al. illustrated the chondro-stimulant role of combined hydrostatic pressure and shear forces in enhancing the secretion of GAG and collagen under in vitro conditions with further chondroprotective effects marked by decreased expression of COL-X.\textsuperscript{144} Frank et al. also described the utility of sinusoidal
Figure 4. The dysfunctioning of the pro-chondrogenic signaling pathways and their mechanistic crosstalk ensued from the vast array of chondrogenic media supplements and the surrounding in vitro biomimetic microenvironment.

macroscopic shear deformation in elevating the proteoglycan synthesis in the cartilage ECM.\[145\] A similar line of reports envisaging the functional importance of biomechanical loading in increasing the ECM deposition as well as enhancing the mechanical stiffness of the engineered cartilage construct have been published by Kisiday et al.,\[146\] Jin et al.,\[147\] and Lee et al.\[148\] However, Ochetta et al. have demonstrated that a 30% confined mechanical compression was sufficient enough to establish hypertrophic maturation in cultured human chondrocytes in a OA-in-chip model.\[149\] Ge et al. also showed that a dynamic compression at an early time point significantly downregulated the chondrogenic marker expressions compared to their static control.\[150\]

A comparative analysis of cyclic dynamic loading versus static loading on the viability of chondrocytes within the bovine cartilage explants revealed enhanced cell death within 3 h of repetitive static loading condition, whereas the cell death was delayed to 6 hours in dynamic cyclic loading.\[151\] Moreover, dynamic loading contributed to a 20% enhancement in GAG synthesis compared to an 84 kPa of static loading condition.\[152\] Furthermore, studies have clearly shown that dynamic compression positively regulated the GAG metabolism in human articular chondrocytes but have a neutral influence on COL-II mRNA expression.\[153\] Additionally, Pigeot et al. also demonstrated the advantage of perfusion bioreactor-based dynamic culture system in promoting concomitant ECM deposition in genetically modified BMSCs.\[154\] Thus, the differential behavior of static and dynamic loading toward chondrogenesis can be attributed to a higher rate of matrix deformation, increased water exudation followed by a change in collagen fiber orientation of the cartilage ECM under static loading conditions inhibiting chondrogenic maturation whereas a negligible matrix deformation and water exudation allow stable chondrogenesis in dynamic loading conditions.\[155\]

The pro-hypertrophic role of hypertensile loading and hydrostatic pressure on human and animal cartilage samples has been extensively studied by Chang et al.\[156\] Results from their investigations revealed the role of Gremlin mediated activation of NF-κB signaling pathway that intensified the production of matrix degrading enzymes (ADAMTS5 and MMP-13), a key hallmark feature of terminally differentiated chondrocytes. A detailed analysis of the underlying signaling cascades behind such ambiguous function of mechanical loading in chondrogenesis revealed the mechanistic crosstalk between a constellation of...
Table 2. Key signaling pathway inhibitors and their role in mitigating chondrocyte hypertrophy.

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Small molecule</th>
<th>Targeted signaling pathway</th>
<th>Inhibition mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Smurf 1/2</td>
<td>Bone morphogenetic protein (BMP)</td>
<td>Degradation of Smad 1 and Smad 5 proteins</td>
<td>[182, 183]</td>
</tr>
<tr>
<td>2</td>
<td>Smad 6/7</td>
<td>Bone morphogenetic protein (BMP)</td>
<td>Formation of heterodimers with Smad 4 via the MH2 domain</td>
<td>[184]</td>
</tr>
<tr>
<td>3</td>
<td>Noggin</td>
<td>Bone morphogenetic protein (BMP)</td>
<td>Blocking the ligand binding sites of both type-1 and type-2 BMP receptors</td>
<td>[161, 139]</td>
</tr>
<tr>
<td>4</td>
<td>Chordin</td>
<td>Bone morphogenetic protein (BMP)</td>
<td>Competitive binding with BMP ligand for the type-1 BMP receptor and inhibit BMP mediated ALP activity</td>
<td>[185, 186]</td>
</tr>
<tr>
<td>5</td>
<td>Dorsomorphin</td>
<td>Bone morphogenetic protein (BMP)</td>
<td>Inhibiting the phosphorylation of Smad 1/5/8 proteins</td>
<td>[187, 188]</td>
</tr>
<tr>
<td>6</td>
<td>LDN193189</td>
<td>Bone morphogenetic protein (BMP)</td>
<td>Inhibiting the phosphorylation of both Smad (Smad 1/5/8) and non-Smad proteins (p38)</td>
<td>[61]</td>
</tr>
<tr>
<td>7</td>
<td>Matrilin-3</td>
<td>Bone morphogenetic protein (BMP)</td>
<td>Interaction with the BMP-2 ligand and mitigating the Smad-1 activity</td>
<td>[189]</td>
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<tr>
<td>8</td>
<td>PKF118-130</td>
<td>Wnt/β-catenin</td>
<td>Preventing the translocation of β-catenin to the nucleus.</td>
<td>[123]</td>
</tr>
<tr>
<td>9</td>
<td>Rofecoxib</td>
<td>Wnt/β-catenin</td>
<td>Blocking the production of Cox-2</td>
<td>[190]</td>
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<tr>
<td>10</td>
<td>XAV-939</td>
<td>Wnt/β-catenin</td>
<td>Phosphorylation mediated degradation of β-catenin through stabilization of AXIN2</td>
<td>[191]</td>
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<tr>
<td>11</td>
<td>Pyruvinium/C113</td>
<td>Wnt/β-catenin</td>
<td>Degradation of β-catenin through activation of casein kinase 1α</td>
<td>[192]</td>
</tr>
<tr>
<td>12</td>
<td>SM04690</td>
<td>Wnt/β-catenin</td>
<td>Inhibition of protease mediated GAG degradation and secretion of inflammatory cytokines and NO.</td>
<td>[164]</td>
</tr>
<tr>
<td>13</td>
<td>Peptide-based inhibitor</td>
<td>Wnt/β-catenin</td>
<td>Suppressing the Wnt mediated transcriptional activity by binding to the TCF docking site of β-catenin.</td>
<td>[193]</td>
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<tr>
<td>14</td>
<td>SAH-Bd9/StAv-33R</td>
<td>Wnt/β-catenin</td>
<td>Preventing the TCF/LEF promoter activity induced by Wnt 3a.</td>
<td>[194]</td>
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<tr>
<td>15</td>
<td>Dkk-1</td>
<td>Wnt/β-catenin</td>
<td>Inhibiting the translocation of β-catenin to the nucleus by internalization of LRPS/LRP6 complex</td>
<td>[195]</td>
</tr>
<tr>
<td>16</td>
<td>Taladegib</td>
<td>Indian Hedgehog (IHH)</td>
<td>Inhibiting the activation of Smoothered protein (Smo)</td>
<td>[196]</td>
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<tr>
<td>17</td>
<td>Curcumin</td>
<td>Indian Hedgehog (IHH) and Notch</td>
<td>Suppressing the expression of Gli2, NICD and Hey proteins</td>
<td>[197]</td>
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<tr>
<td>18</td>
<td>G141</td>
<td>Fibroblast Growth Factor (FGF)</td>
<td>Competitively blocks the activation of FGF receptor through interaction with its non-ATP binding pockets</td>
<td>[167]</td>
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<tr>
<td>19</td>
<td>Bevacizumab</td>
<td>Vascular Endothelial Growth Factor (VEGF)</td>
<td>Blocking the initiation of angiogenesis during hypertrophic maturation</td>
<td>[198]</td>
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<tr>
<td>20</td>
<td>Gefitinib</td>
<td>Epidermal Growth Factor (EGF)</td>
<td>Inhibiting the phosphorylation mediated activation of EGFR</td>
<td>[168]</td>
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<tr>
<td>21</td>
<td>Xanthotoxin</td>
<td>p38 Mitogen Activated Protein Kinase (MAPK) /Histone deacetylase 4 (HDAC4)</td>
<td>Downregulating the phosphorylation of p38 and elevating the expression of HDAC4</td>
<td>[199]</td>
</tr>
<tr>
<td>22</td>
<td>Asiatic acid</td>
<td>AMP-activated protein kinase (AMPK) /Phosphoinositide-3 kinase/Protein kinase B (PI3K/AKT)</td>
<td>Phosphorylation mediated activation of AMPK with simultaneous decrease in phosphorylation level of PI3K and AKT</td>
<td>[200]</td>
</tr>
<tr>
<td>23</td>
<td>U0126</td>
<td>Mitogen activated protein kinase (MAPKKK)/extracellular signal regulated kinase (ERK)</td>
<td>Inhibiting the activation of ERK 1/2 by suppressing the kinase activity of MEK.</td>
<td>[201]</td>
</tr>
<tr>
<td>24</td>
<td>Cordycepin</td>
<td>Phosphoinositide-3 kinase (PI3K)/Bapx1 and Notch</td>
<td>Downregulating the expression of PI3K (hypertrophy suppression) and inhibiting nrf2 expression (chondrogenesis stimulation)</td>
<td>[202]</td>
</tr>
<tr>
<td>25</td>
<td>Kartogenin</td>
<td>c-Jun N-terminal kinase (JNK) / β-catenin</td>
<td>Increasing the phosphorylation level of JNK while suppressing the β-catenin mediated transcription activity.</td>
<td>[203]</td>
</tr>
</tbody>
</table>
hypertrophy stimulating inflammatory and non-inflammatory
signaling pathways like TNF-α, IL-1β, NF-κB, Wnt, and TGF-
β. Thus, it can be rightly hypothesized that an improper balance in degree of application of mechanical loading as well as
the time and point of application could possibly shift the carti-
lage homeostasis paradigm from pro-chondrogenic to hypertro-
phy stimulating form, leading to the formation of a transient carti-
lage phenotype or bone (Figure 4).

7. Measures Adopted to Halt Chondrocyte
Hypertrophy: Should we Completely Block or
Apply a Brake?

Small molecule regulators of pro-hypertrophic signaling path-
ways: A wide array of small molecules has been deployed by re-
searchers and clinicians worldwide to suppress the hypertrophic maturation of chondrocytes with a common objective to develop a phenotypically stable articular cartilage while suppressing chondrocyte hypertrophy. One potent inhibitor of BMP signaling pathway is LDN193189 that arrests the colocalization of SMAD-4 protein with the SMAD-1/5/8 BMP downstream target as evident from the downregulated expression of hypertrophy inducing genes IHH, COL-X and MMP-13 when incubated with an OA microcartilage model (Figure 6). Several other natural (Chordin, Noggin) and synthetic (K02288) BMP inhibitors have also demonstrated impeding expression pattern of IL-1β and BMP-2 protein with redundant ALP activity through competitive inhibitory binding mechanism with the BMP ligands. Similarly, PKF118-310 and SM04690 targets the pro-
hypertrophic version of Wnt signaling pathway by mitigating the expression of hypertrophy inducing targets of Wnt pathway besides promoting the expression of chondrogenic marker genes (COL-II, ACAN, and SOX-9). The inhibition of IHH pathway by Ipriflavone and Cyclopamine has been highly effec-
tive in abrogating hypertrophic differentiation by reduced expres-
sion profile of IHH pathway associated genes SMO and Gli2 besides the redundant expression of osteogenic marker RUNX2, MMP-13 and COL-X. A variety of other inhibitors have been re-
ported like Bevacizumab as an anti-VEGF antibody, G141 as a suppressor of Fibroblast growth factor (FGF) pathway and Gefitinib as an inhibitor to Epidermal growth factor receptor
Figure 6. Immunostaining of the developed OA microcartilage model using BMP-2 induction of human articular chondrocytes. The enhanced expression of chondrocyte hypertrophic marker MMP-13 and COL-X have been substantially reduced with the treatment of BMP signaling pathway inhibitor LDN193189 and Compound A (developed by Novartis). Reproduced with permission. Copyright 2020, The Company of Biologists.

(EGFR), significantly downregulated the overexpression of matrix degradation components (MMP-1/13) along with enhanced collagen and GAG synthesis in vitro details of which are included in Table 2. But the major challenge lies in the limited half-lives of such small molecules, for example, LDN193189 has a half-life of 1.5 hours. Spatial tethering and controlled release strategy of such small molecules through polymeric biomaterial would enhance their half-life, cell specificity, bioadhesivity, and active transportation to cellular receptors.[169] (Figure 5)

However, the critical question to raise is whether to completely inhibit these signaling cascades or draw a balance through their time-dependent dosage? Reports from different research groups have shown the pro-chondrogenic activity of TGF-β in regulating the entire process of cartilage development right from stimulating mesenchymal condensation to chondrogenic differentiation and further inhibiting transient cartilage differentiation. However, research from our group demonstrated a 21-day exposure of TGF-β1 to the BMSCs triggered hypertrophic differentiation.[112,170] A similar paradoxical relationship of BMP with chondrogenesis have been illustrated where BMP protein significantly enhanced the intracellular communication through N-cadherin expression in one hand and the overexpression of chondrocyte hypertrophic marker genes (RUNX2, COL-I, COL-X, and ALP).[24,51] Hypoxia is also known to exhibit a dual functionality in chondrogenesis where HIF-1α promotes chondrogenic differentiation and HIF-2α elevates hypertrophic maturation of chondrocytes. The canonical Wnt signaling pathway is one of the key regulators in the transition from a healthy chondrocyte to a hypertrophic chondrocyte phenotype. In an animal model, the expression of Wnt 5a in was observed in the early chick perichondrium whereas the same Wnt ligand further inhibited the hypertrophic transition.[123,171] Hence, instead of a long-term exposure to a particular biochemical mediator, an in vitro acceleration or retardation of this transition would result in direct stimulation or inhibition of endochondral ossification. Similarly, the unanimous Wnt14 expression both as the molecular marker for earliest inducer of joint formation as well as in the MSCs of the cartilage tissue necessitates the development of stable cartilage tissue phenotype simulating the in vivo anatomy and physiology.[172]

Due to the enigmatic role of these signaling pathways in regulating stable chondrogenesis, a complete inhibition might compromise the development of chondrogenic phenotype during the process of chondrogenic differentiation. Thus, drawing a brake by applying stringent control over the exposure time and concentration of these signaling molecules can create a balance in up-regulation and downregulation of respective signaling cascades and thereby allow the development of stable articular cartilage phenotype.
8. Conclusion

The current review focused on comprehending the underlying mechanism behind the occurrence of chondrocyte hypertrophy during the expansion and in vitro chondrogenesis of stem cells (BMSCs, ADSCs) and primary chondrocytes. We formulated four hypotheses to be the major causative reasons: a) the acquisition of de-differentiated phenotype by primary chondrocytes during expansion as one of the contributors to hypertrophy phenotype, b) the intrinsic property of stem cells to undergo hypertrophic maturation under the influence of chondrogenic stimulants through the dysregulated activation of chondrogenic signaling pathways, c) the aberrant role of hypoxia, and d) the imbalance in mechanical loading conditions during in vitro chondrogenesis leading to the formation of a transient cartilage over a permanent one. Literature-based evidence have been provided for each hypothesis to elucidate the redundancy and their significant outcome in causing hypertrophic maturation following chondrogenesis in vitro. But it is still a matter of comprehensive research whether chondrocyte hypertrophy is an active driver of OA or a passive consequence of OA.

Finally, while describing the different strategies in minimizing chondrocyte hypertrophy, a question was raised as to whether completely inhibit the pathways leading to hypertrophy or apply a strict checkpoint to ensure a proper balance between chondrogenesis and hypertrophy. Such balance will successfully allow stable chondrogenesis without interfering in the embryonic endochondral ossification route. The transient cartilage differentiation in the engineered or implanted cartilage would result in a modified tissue microenvironment (significantly different from native articular cartilage), which blocks phenotypically stable articular cartilage differentiation. In order to neutralize this transient cartilage phenotype, a comprehensive tissue regeneration protocol/approach should be devised augmenting controlled release of small molecules (e.g., zone-specific Wnt inducers or BMP inhibitors) from 3D Bioprinted cartilage construct to achieve similar differentiation program akin native articular cartilage during embryonic development. The resultant phenotypically stable tissue engineered articular cartilage would circumvent all the problems associated to non-optimal consistency and can be used as a regenerative therapeutic strategy for the prevailing osteoarthritic patients by the clinicians worldwide.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

chondrocyte hypertrophy, chondrogenesis, de-differentiation, permanent versus transient cartilage, stem cells
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