The Good the Bad and the Ugly of Glycosaminoglycans in Tissue Engineering Applications

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Abstract: High sulfation, low cost, and the status of heparin as an already FDA- and EMA-approved product, mean that its inclusion in tissue engineering (TE) strategies is becoming increasingly popular. However, the use of heparin may represent a naïve approach. This is because tissue formation is a highly orchestrated process, involving the temporal expression of numerous growth factors and complex signaling networks. While heparin may enhance the retention and activity of certain growth factors under particular conditions, its binding ‘promiscuity’ means that it may also inhibit other factors that, for example, play an important role in tissue maintenance and repair. Within this review we focus on articular cartilage, highlighting the complexities and highly regulated processes that are involved in its formation, and the challenges that exist in trying to effectively engineer this tissue. Here we discuss the opportunities that glycosaminoglycans (GAGs) may provide in advancing this important area of regenerative medicine, placing emphasis on the need to move away from the common use of heparin, and instead focus research towards the utility of specific GAG preparations that are able to modulate the activity of growth factors in a more controlled and defined manner, with less off-target effects.

Keywords: glycosaminoglycans; heparin; heparan sulfate; cartilage; mesenchymal stem cells; tissue engineering; growth factors; growth differentiation factor 5; GDF5

1. Introduction

Tissue Engineering (TE) is a multidisciplinary research area involving combinations of biomaterials, differentiating cells, and bioactive factors to produce functional tissues and organs. Articular cartilage has limited ability to regenerate, and has therefore become a key target for TE strategies. However, despite sustained efforts, most techniques to date have failed to generate a truly biomimetic articular cartilage. Here we review the biology behind this highly structured tissue, the challenges that have been faced in trying to engineer cartilage, and the advancements that are currently being made within this field. In particular, we highlight the exciting potential that incorporating GAGs into cartilage TE strategies may provide, as well as the limitations and drawbacks that they may introduce if not used in a controlled manner.
2. Articular Cartilage

Articular (hyaline) cartilage is a predominantly alymphatic, aneural, and avascular tissue, consisting of chondrocytes, which make up only 1–5% of its total volume, embedded within an extensive extracellular matrix (ECM) (as reviewed [1–3]). Being widely dispersed, chondrocytes are responsible for the synthesis, maintenance and turnover of the ECM, in response to signals from growth factors, cytokines, adipokines, inflammatory mediators and matrix fragments (see [4]). Type II collagen and the proteoglycan (PG) aggrecan, play the most important structural roles in the ECM of cartilage, together forming a hydrodynamic, tensile meshwork, with a high compressive strength [2,5]. Given these properties, the tissue plays a vital role in load-bearing joints, and provides an almost frictionless surface to articulating bones [6]. The ECM of articular cartilage is also highly structured and organised, and can be divided into four spatially distinct regions, namely the superficial, middle, deep, and calcified zones (see [6–8]). As shown in Figure 1, each zone is characterised by unique ECM compositions, mechanical properties and cellular organisation. Studies have demonstrated that even small changes in the ECM of articular cartilage can lead to disruption in its mechanical properties, highlighting that structural organisation and continual remodelling of ECM molecules by chondrocytes is crucial to the proper functioning of the tissue [9,10].

![Figure 1](image-url)  
**Figure 1.** Schematic demonstrating the layered structure of articular cartilage. The transition from the superficial to the calcified zone is characterised by an increase in GAG content and compressive strength, but a decrease in collagen II. Collagen X is usually only found in the calcified zone of healthy articular cartilage. Figure adapted from [8,11]. Figure not to scale.

2.1. Formation

In many respects, our understanding of the mechanisms involved in the formation, organisation and maintenance of articular cartilage remain unclear and challenging [12]. Much of the progress that has been made to date has come from research into the formation of the axial and appendicular skeletons, which is initiated by limb bud development, through the highly regulated process of endochondral ossification [13–19]. This process begins early during embryogenesis with the aggregation and condensation of mesenchymal stem/stromal cells (MSCs) derived from the mesoderm germ layer. MSCs within these condensations then proliferate, increasing the cell density within the condensate, before undergoing differentiation into prechondrogenic cells that form a cartilage template/anlagen. At this stage the prechondrogenic cells are then thought to either further differentiate into chondrocytes that produce large amounts of ECM and form permanent hyaline cartilage, or into proliferating chondrocytes that form the growth plate and eventually undergo hypertrophy [19] (Figure 2A).
Figure 2. Articular cartilage formation. (A) Chondrogenesis is initiated during limb bud development with the condensation of MSCs. These progenitors then differentiate into chondrocytes that go on to form permanent articular cartilage, or into chondrocytes that eventually undergo hypertrophy and endochondral ossification. The complex spatiotemporal cues required to maintain chondrocytes in a permanent articular cartilage-like phenotype are not yet fully understood, and as such, the majority of regeneration strategies currently result in the formation of hypertrophic rather than hyaline-like tissue; (B) Articular cartilage is thought to originate from a distinct population of MSCs during limb joint formation; GDF5/Erg/Gli3 expressing cells within the joint space define the initial interzone MSC population, and this population becomes sandwiched between the two cartilage anlagen, while anlagen bound chondrocytes turn on expression of Matn1. GDF5/Erg/Gli3 expressing cells adjacent to their respective cartilaginous anlagen, but which have never expressed Matn1, then go on to differentiate into articular chondrocytes. (A) adapted from [20]; (B) adapted from [12]. Abbreviations: Dcx, doublecortin; OA, osteoarthritis.

Accumulating evidence suggests that permanent hyaline cartilage originates from a distinct population of MSCs, referred to as interzone cells (see [12,21]). These cells are found sandwiched between cartilage anlagen and are characterised by expression of genes such as erythroblast transformation-specific-related gene (Erg), growth differentiation factor 5 (GDF5), and GLI family zinc finger 3 (Gli3) [22–24] (see Figure 2B). Cell lineage tracing experiments have shown that as interzone cavitation and joint capsule formation occurs, articular cartilage and other synovial joint components are originated specifically from this interzone population of cells [24–26]. Notably, Hyde et al. [26], have shown that, unlike the remainder of chondrocytes in the cartilage anlagen, articular chondrocytes are derived from a specific group of chondrocytes, which have never expressed matrilin-1 (Matn1). In
contrast, pre-chondrocytes found at secondary ossification sites at the edge of cartilage entities are instead transient in nature, proliferating and increasing in size before undergoing hypertrophy and contributing to bone growth [22]. Hypertrophic chondrocytes exit the cell cycle, secrete a distinct ECM rich in collagen X, and can be characterised by the expression of terminal markers of hypertrophic differentiation such as runt-related transcription factor 2 (Runx2), matrix metalloprotease 13 (MMP13), Indian hedgehog (Ihh), and collagen X [27–31]. Mineralization and blood vessel formation are also hallmarks of the phenotype [32]. Until recently, it was thought that the hypertrophic chondrocytes eventually undergo apoptosis allowing the infiltration of osteoblasts and bone formation, however, new evidence suggests some hypertrophic chondrocytes can survive the transition and become osteogenic cells [33]. Although evidence suggests that articular chondrocytes have a distinct embryonic origin from those that form the growth plate, under pathological conditions such as osteoarthritis (OA), chondrocyte hypertrophy is reactivated leading to calcification and outgrowths of bone, indicating that permanent articular chondrocytes can also acquire features of a more transient phenotype [34,35].

Chondrocyte hypertrophy is also a common outcome of in vitro and in vivo cartilage TE/regeneration strategies [30,36–41]. TE strategies must therefore focus on creating ‘permanent’ cartilage, which is able to maintain its structure and function throughout life and withstand hypertrophic differentiation [42] (see Figure 2A). A clearer understanding of the mechanisms and factors that trigger and suppress entry into the hypertrophic differentiation pathway will therefore be key to the success of future approaches.

2.2. Disease and Trauma

The low cell density and avascular nature of articular cartilage means that the tissue has limited potential for regeneration and repair following injury [3,43]. Instead, when a traumatic lesion, or degenerative disease occurs, the defect is filled with fibrous tissue, which is often unable to withstand the compressive and shear forces which act upon the joint, leading to cartilage breakdown, pain and immobility (as reviewed [3,43]). Cartilage damage can occur as a result of traumatic injuries such as ligament tears, impact, joint dislocation, infection and inflammation. While biomechanical loading is necessary for articular cartilage homeostasis, abnormal, or altered loading is associated with inflammation, metabolic imbalances and joint instability (see [44]). Damage can also occur gradually as the result of degenerative joint diseases such as OA, characterised by pain, structural changes, gradual loss of articular cartilage, and eventual direct bone-bone contact and joint destruction [45]. In OA it is still unclear whether it is the chondrocytes that drive cartilage pathology, or whether they are just responding to the disease process from elsewhere in the joint [46]; the balance between these processes may differ between patients and disease subgroups. OA is an epidemic problem and a major cause of decreased quality of life in adults [47]. It is estimated that one third of people over the age of 45 have sought treatment for OA in the UK [48], and this number is expected to rise in line with the ageing population and increasing obesity problem [49]. Therefore, there is a great need for the development of new approaches to prevent and treat cartilage damage.

2.3. Current Therapies

While knee-replacement surgery is becoming an increasingly common procedure, with around 97,000 replacements carried out each year in the UK alone [50], treatment of cartilage injury at an earlier stage to postpone or avoid total joint replacement would be preferable. Current treatments used in the clinic for cartilage damage include mosaicplasty, autologous chondrocyte implantation (ACI), and microfracture, each of which have shown various degrees of success, but which prove typically unsatisfactory in the long term [51,52]. For example, in the case of mosaicplasty, damaged cartilage is replaced with cartilage plugs (allo- or auto-grafts), which are poor at integrating with the host tissue, and in the case of allografts, have the potential for disease transmission [53]. ACI was first described in 1994, and involves the use of autologous chondrocytes, which are isolated from an uninjured area of the knee, expanded, and injected into the area of the defect [54]. The procedure has since been adapted to include the use of collagen/hyaluronan (HA) scaffolds (matrix-induced ACI
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(MACI)), allowing for improved cell attachment and outcomes [55,56]. However, numerous limitations bedevil ACI/MACI outcomes, including donor site morbidity, the amount of time taken to expand the cells ex vivo, the inability to treat large cartilage defects, expense, and patient disability for long periods of time before surgical implantation can take place [51,57]. In addition, adult chondrocytes are known to de-differentiate during the expansion process, losing their spherical morphology and ability to synthesize aggregan and collagen II [58,59]. Following implantation, hypertrophic growth has also been associated with these grafts [60]. Chondrocytes of older patients also have age-related intrinsic changes, such as decreased mitotic activity and telomere length [61,62]. As already mentioned, it is also unclear whether degenerative diseases such as OA are an ageing disorder of the chondrocyte itself, or whether chondrocytes are simply responding to the disease process initiated elsewhere [46]. Ultimately, therapeutic options for articular cartilage repair remain insufficient, despite the increasing prevalence of cartilage disease. While the use of chondrocytes in ACI/MACI procedures has marked the first steps in repairing damaged cartilage, with an ageing population the need for a more reliable, efficient and durable method of cartilage repair is clear.

3. Mesenchymal Stem Cells

Stem cells have become an attractive alternative to the use of chondrocytes for cartilage TE strategies, due to their relative abundance, self-renewal, and multipotent or pluripotent capabilities, therefore avoiding many of the limitations of chondrocytes [63–65]. The best stem cell source for cartilage TE is yet to be identified, with MSCs, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), all being considered due to their individual strengths and weaknesses (as reviewed [66]). Ease of isolation, differentiation potential, surface marker expression and cost are just some of the factors that must be considered. An obvious benefit of ESCs and iPSCs is their therapeutic flexibility and continuous self-renewal capabilities. However, the use of ESCs carries high ethical and political concerns, the possible need for immunosuppressants, and the issue of tumorigenicity [67–69]. iPSCs also carry a high risk of teratoma formation, show low reprogramming efficiencies, and have been found to retain the epigenetic memory of their somatic cell of origin [70–72]. While the use of ESCs and iPSCs may become particularly important for the regeneration of tissues that cannot be repaired by alternative cell types, their limitations have meant that human MSCs (hMSCs) have remained a particularly attractive cell source for cartilage (and other) TE strategies [73,74]. hMSCs are relatively easily available, can be extracted from multiple tissue sources in relatively large quantities with limited morbidity, are capable of self-renewal, lack tumorigenicity, are applicable to autologous transplantation procedures, and can differentiate into multiple cell lineages, including chondrocytes [75–77]. MSCs are also known to secrete soluble cytokines and growth factors in a paracrine fashion, which are thought to add to their therapeutic effects [78]. In addition, MSCs have been indicated to have stronger immunomodulatory properties compared to other stem cell sources, and have been shown to suppress T cell, B cell, dendritic cell and natural killer cell activity [75,79–82]. MSCs are therefore not only attractive for tissue regeneration via their multilineage differentiation potential, but also for treatment of autoimmune diseases such as rheumatoid arthritis (RA), and to help bypass graft-versus-host disease (GVHD) [83–86]. In addition, MSCs have the obvious benefit over ESCs and iPSCs for cartilage TE, that they are already committed to mesoderm lineages [87], and therefore require less in vitro programming.

hMSCs were first used in a clinical trial to treat full thickness cartilage defects in 2004 [88], and have since been included in an increasing number of clinical trials [89–92]. Generally, results suggest that hMSCs are a promising cell source for cartilage repair, but there is a general lack of comparative studies and systematic reviews, and much remains to be investigated and optimised (see [73]). Some of these issues have been outlined below.
3.1. Isolation and Characterisation of MSCs

Adult MSCs are derived from the mesoderm and reside in the adult body throughout life, generally decreasing in abundance with age [87,93,94]. MSCs were first isolated from bone marrow [95,96], but have since been extracted from numerous sources including, adipose tissue [97], skeletal muscle [65], the synovium [98], and the umbilical cord [99]. The inherent differences among the microenvironments of each of these stem cell populations means that although each share similar phenotypic and functional properties, differences in differentiation capacities, and surface marker expression do exist [100,101]. This review will focus on bone marrow-derived MSCs (BMMSCs), which are the most well characterised and commonly used adult stem cell source for cartilage TE [102]. However, it is important to note that synovial membrane derived MSCs (SMSCs) are also becoming an interesting alternative due to their proximity to articular cartilage and tissue-specific properties for connective tissue repair [103–105]. Adipose derived MSCs are also gaining increased interest due to their abundance (5% of nucleated cells versus 0.0001–0.01% for BMMSCs) and the ease with which they can be harvested [106,107].

BMMSCs are isolated from the mononuclear layer of the bone marrow and are characterised by their fibroblast-like morphology, proliferation to form loose colonies of spindle shaped cells, and ability to adhere to tissue culture plastic [108,109]. The cells are passaged when they reach 80–90% confluence, with serial passages and washes generally thought to remove any non-MSC types. However, it is important to note that the resulting cultures are still heterogeneous and this will be discussed in the following section.

Phenotypically, no specific markers for hMSCs have been identified to date. Instead characterisation is based upon a panel of positive and negative markers. Generally, it is considered that hBMMSCs are negative for the hematopoietic markers CD45, CD34, CD14 and CD11, as well as for the co-stimulatory markers CD80, CD86 and CD40, and the adhesion molecules CD31, CD18 and CD56 [80]. The cells are also characterised as being positive for CD105, CD73, CD44, CD90, CD71 and Stro-1, as well as for the adhesion molecules CD106, CD166, ICAM-1 and CD29. Importantly, MSC surface markers have been shown to vary between species, tissue sources, and methods of isolation and culture. For example, a study by De Ugarte et al. [110], noted differences in expression of the cell adhesion molecules CD49d, CD54, CD34, and CD106, between MSCs isolated from bone marrow and adipose tissue. This highlights the need for a more uniform characterisation of MSCs, as it is becoming increasingly difficult to compare and contrast findings between investigators who are likely to be looking at distinct/different cell populations. A further way to identify MSCs is through their multilineage potential to differentiate into adipocytes, chondrocytes and osteoblasts [111]. However, the plasticity of MSCs has also come under further investigation, as studies have shown that the cells are also capable of differentiating down the myogenic and neuronal lineages [112–115].

In 2006 the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) decided to try and address the inconsistent use of defining characteristics, and lack of universally accepted requirements to define MSCs [111]. It was proposed that the minimal requirements to define hMSCs would be: (i) the ability to adhere to culture tissue plastic; (ii) to express CD105, CD73 and CD90; (iii) to lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface markers; (iv) and to differentiate into osteoblasts, adipocytes and chondrocytes. Although these guidelines have provided the first steps in streamlining the characterisation of hMSCs, further refinements are clearly required if their full therapeutic potential is to be met [116–120]. Ultimately, the ubiquitous term of ‘MSCs’ remains one of the most undefined and controversial areas in stem cell research. It has even been suggested that this terminology should be abandoned altogether, with the argument that ‘MSCs’ with identical differentiation capacities do not exist [121].

3.2. MSC Heterogeneity

The low frequency of MSCs within human tissue means that the cells require extensive ex vivo expansion before they can be used for in vitro/in vivo testing, or indeed for potential clinical
application. However differences in isolation methods, culture conditions and media additives used between different laboratories means that variability in cell yield and the phenotype of the expanded cell products are inevitable [122,123]. As well as improved characterisation of MSCs, standardisation of the procedures used for their isolation and expansion should therefore also be a priority, so that comparisons between studies can be made more effectively. MSCs are known to display a high degree of heterogeneity, and outgrowth of certain subsets of cells driven by the differences in culture conditions is likely to promote or inhibit their differentiation potential [124]. A better understanding of how this is controlled, and which conditions promote the differentiation of MSCs into certain cell types, is clearly needed. In the case of cartilage, better understanding of what drives permanent articular-cartilage formation, rather than fibrocartilage or hypertrophic differentiation is of the utmost importance to their success in TE/regeneration strategies [125–127]. Torensma et al. [124] suggest that the leading cause of heterogeneity within MSC cultures is, unsurprisingly, the tissue source, followed by the culture methodology, and then individual donor variation. Remarkably, in the same study, it was also shown that expanded cells which were then frozen and distributed to different laboratories to be grown for one passage, also developed some heterogeneity; indicating that cell culture location also has an effect.

3.3. In Vitro Chondrogenic Differentiation of MSCs

The most common and established method for in vitro chondrogenic differentiation of BMMSCs is often referred to as pellet culture and was developed by Johnstone et al. [36] using rabbit BMMSCs. The group then went on to use the same technique with hBMMSCs [37]. Briefly the technique involves spinning 200,000 cells at 500 g in 15 mL polypropylene conical tubes to form spherical cell aggregates. The spheroids are cultured in defined, serum-free medium consisting of high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with transforming growth factor beta 1 (TGFβ1), ascorbate-2-phosphate, sodium selenite, transferrin, dexamethasone, insulin, and sodium pyruvate. The technique is highly reproducible, with cell aggregates being harvested at time points up to 28 days, and some groups adapting the protocol to use up to 500,000 cells per aggregate [59]. Mackay et al. [128] went on to show that the addition of TGFβ3 rather than TGFβ1 better supports chondrogenesis.

The simplicity of the pellet culture method means it has remained popular for examining the various signalling pathways and soluble factors involved in chondrogenic differentiation, as well as for disease modelling [129–131]. However, mass transport limitations mean that the pellet cultures are not made up of a homogenous population of cells, and ECM production is not as extensive as you would hope for in an articular cartilage-like tissue, especially towards the centre of constructs [132]. In addition, the spheroid geometry of the pellet culture does not match the structured organisation of cartilage in vivo [7,8].

Murdoch et al. [41], modified the pellet culture method, introducing a porous membrane as a support for the cells. The method uses the same media and supplements as mentioned above, but the cells (500,000) are seeded onto Transwell filters and spun in a 24-well plate at 200 g, creating a shallow multilayer of MSCs that are then able to grow and differentiate into a disc of cartilage-like tissue [41,59,133]. The Transwell culture method has been shown to lead to more rapid and efficient differentiation of MSCs, and the deposition of a more extensive cartilage-like ECM when compared to the pellet culture system [41]. In addition there is a more uniform differentiation of the MSCs with improved mass transport within the shallow and permeable disc geometry [59]. Recently, it has also been shown that the Transwell system allows for the production and assembly of organised and cross-linked collagen networks; helping to explain the robust, flexible nature of the constructs that are formed [133]. However, despite the clear improvements in this technique, many researchers are still using the pellet culture method, perhaps due to the difficulty in reproducing the Transwell method effectively.

These difficulties and limitations have meant that the use of scaffolds/biomaterials to improve the in vitro chondrogenic differentiation of MSCs has become a popular avenue of research, allowing
for a better recapitulation of the in vivo environment and production of larger scale cartilaginous constructs that are more clinically relevant. Ultimately though, despite much progress over the last two decades, current methods for the in vitro chondrogenic differentiation of hMSCs are still a long way off reaching the clinic. Indeed, as far as we are aware, pre-differentiated MSCs have not yet been used in any clinical trials for cartilage repair. Perhaps the most pressing concern/limitation regarding the application of pre-differentiated hMSCs is that of chondrocyte hypertrophy, with most differentiation protocols resulting in the formation of chondrocytes with endochondral-like maturation properties rather than a true articular cartilage-like phenotype [36–38,41,134–137]. In contrast, the use of undifferentiated hMSCs for cartilage repair has progressed further, with around 50 clinical trials currently registered at https://www.clinicaltrial.gov/. Published results have generally been promising, and the use of allogenic umbilical cord MSCs have already been approved by the Korean FDA for OA treatment [92,126,138–144]. However, the use of these cells for cartilage formation is still in its infancy, and from the evidence that is available, protocols to improve the quality of the regenerated cartilage are still clearly required [88,126,145–147]. As such, there is an overriding necessity to better understand the mechanisms controlling permanent articular cartilage formation, alongside further evaluation of ongoing clinical trials, before these cells can be used to their fullest potential in the clinic. Whether the greatest success will be seen with the in vitro chondrogenic differentiation of hMSCs, in vivo delivery of undifferentiated cells, or with cell-free approaches that instead harness the body’s own MSCs for repair, remains to be seen. However, what is clear is that the use of current in vitro models (such as the pellet culture) to screen potential bioactive factors (such as growth factors) and culture conditions, as well as investigation into biomaterials that can help support articular cartilage formation, should be a priority, and will be of the upmost importance for the improved development of both in vivo and in vitro regeneration strategies.

4. Growth Factors Involved in the Chondrogenic Differentiation of MSCs

The differentiation of MSCs into chondrocytes involves the activation and suppression of a number of signalling pathways and growth factors, with members of the TGFβ, fibroblast growth factor (FGF), and insulin-like growth factor (IGF) families, among others, having all been implicated in the process (as reviewed in Table 1). The TGFβ superfamily has been the most researched of all stimulating factors in the area of chondrogenesis, and is described in more detail in the sections below.

| Molecule Family | Molecule | Proposed Function during Chondrogenesis | Reference | Associated PGs/GAGs | Reference |
|-----------------|----------|----------------------------------------|-----------|---------------------|----------|
| FGF 1           | FGF2     | Enhances proliferation and chondrogenic potential during expansion. | [59,148–151] | Role of HSPGs in FGF-receptor binding has been extensively studied; HSPGs play an important role in FGF-receptor signalling by facilitating ligand-receptor oligomerisation. | [155–159] |
|                 |          | Negative effect on matrix deposition and differentiation. | [151–153] | | |
|                 |          | Addition during expansion primes cells for hypertrophy. | [149,151] | | |
|                 |          | Prolongs lifespan of MSCs. | [154] | | |
| FGF9            | Increases matrix production early on, but then promotes matrix resorption and hypertrophy. | [152] | CS sulfation patterns have also been implicated in articular cartilage formation and expression has been co-localised with FGF2. Perlecan can only deliver FGF2 to its receptors after its CS chains have been removed. | [160–162] |
|                 | However, also reported to promote matrix production and delay terminal hypertrophy. | [151] | | |
| FGF18           | Suppresses proliferation and promotes matrix production. | [151,164] | Exogenous HS can be used to improve hMSC expansion. | [163] |
|                 | Delays terminal hypertrophy. | [151] | | |
### Table 1. Cont.

| Molecule Family | Molecule | Proposed Function during Chondrogenesis | Reference | Associated PGs/GAGs | Reference |
|-----------------|----------|----------------------------------------|-----------|---------------------|-----------|
| TGFβ            | TGFβ1/3  | Promotes chondrogenic differentiation of MSCs. Considered a main chondrogenic inducer of MSCs, however, leads to chondrocyte hypertrophy. | [36–39,41,75,128,165] | TGFβ1 but not TGFβ3 has been shown to bind to HS; effects of the interaction remain conflicting. | [166–169] |
|                 |          | TGFβ3 better supports chondrogenic differentiation than TGFβ1. |           | TGFβ binds to the small leucine rich PGs, decorin, biglycan and fibromodulin, but via their protein core; CS/DS chains interfere with this binding. | [170] |
| GDF5            |          | Important role in joint formation and organisation of articular cartilage; GDF5 expressed in healthy pre-hypertrophic cartilage, but not as OA develops; GDF5 dominant negative mutation results in articular cartilage degeneration. | [23,171–175] | Heparin binding sequence predicted. | [182] |
| BMP             |          | Increases cartilaginous ECM production in vitro. | [176–179] | Use of heparin/HS to potentiate the activity of BMPs has been widely studied; especially in the case of BMP2 for bone TE. | [187–195] |
| BMP2/4/6/7      |          | Promotes chondrogenic differentiation of MSCs, especially when used in combination with TGFβ. BMP2/7 indicated as particularly useful for inducing chondrogenesis. However, most studies indicate that BMP supplementation also leads to hypertrophy. | [153,181,183–186] | Glypican3 is strongly linked to the Wnt pathway; HS chains bind to Wnts with different affinities to fine-tune access to Wnt receptors; 6-O-sulfation of HS reduces ability of Glypican1 HS chains to bind Wnt, and therefore facilitates Wnt-receptor interaction. | [196–198,206] |
| Wnt3a, Wnt5a    |          | Promotes chondrogenic differentiation. Inhibits hypertrophy. However, Wnt5a also reported to promote hypertrophy during early stages of differentiation. | [196–198,199] | Glypican3 is strongly linked to the Wnt pathway; HS chains bind to Wnts with different affinities to fine-tune access to Wnt receptors; 6-O-sulfation of HS reduces ability of Glypican1 HS chains to bind Wnt, and therefore facilitates Wnt-receptor interaction. | [200–202] |
| Wnt11           |          | Promotes chondrogenic differentiation and hypertrophy. | [203] | Heparin/HS/DS stimulate the release of free and bioactive IGF1 from IGF binding proteins. | [207] |
| Wnt4, Wnt8      |          | Inhibits chondrogenic differentiation. Promotes hypertrophy. | [196,204] | | |
| Wnt9a           |          | Inhibits chondrogenic differentiation. Inhibits hypertrophy. | [205] | | |
| IGF             | IGF1     | When used in combination with TGFβ3 collagen I production is reduced. Promotes hypertrophic differentiation. | [183,206] | Heparin/HS/DS stimulate the release of free and bioactive IGF1 from IGF binding proteins. | [207] |
| PTHrP           | PTHrP (1–34) isofrom | Inhibits TGFβ induced hypertrophic differentiation. | [39,153,206,209] | PTHrP is activated by Ihh signalling (feedback loop). HS binds Ihh and negatively regulates signalling. | [210,211] |

1 FGF, fibroblast growth factor; HSPG, heparan sulfate proteoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; TGFβ, transforming growth factor beta; BMP, bone morphogenetic protein; GDF, growth differentiation factor; Wnt, Wingless-type MMTV integration site; IGF, insulin like growth factor; PTHrP, parathyroid hormone-related peptide.
4.1. Transforming Growth Factor Beta (TGF\(\beta\)) Superfamily

The TGF\(\beta\) superfamily of secreted factors is made up of more than 30 members that can be phylogenetically split into two main groups, namely the TGF\(\beta\)/Activin and the BMP/GDF sub-families [212,213]. Members of the family are synthesised as large precursor (inactive) molecules, which then undergo proteolytic cleavage following dimerisation, to yield active, mature dimers [214]. All mature monomers of the family share a conserved cysteine knot structure, formed from six characteristically spaced disulfide-bonded cysteines within the C-terminal region [215,216]. In addition, with the exception of GDF3 and GDF9, members of the family contain a seventh conserved cysteine residue, which is required for the covalent linking of the dimeric structures [217]. Upon secretion, the active mature dimers are then able to bind to their respective receptors to initiate downstream signalling, although a number of further regulatory mechanisms exist, such as the secreted antagonists Noggin and Chordin [218], and where ECM molecules such as heparan sulfate (HS) also play a role [169]. In addition, it has been shown for some TGF\(\beta\) superfamily members (such as TGF\(\beta\)1/2/3 and GDF8), that the mature ligand is secreted from cells in a large complex which includes the cleaved pro-region, known as the latency-associated protein (LAP) (see [213,219]). These complexes have also been shown to bind to latent TGF\(\beta\)-binding proteins (LTBPs), which are structurally related to fibrillins and allow for ECM localisation [220]. The complexes remain inactive in the ECM, until the mature ligand is released from the complex by activators such as integrin receptors, proteases, or reactive oxygen species [220–225]; thereby adding another level of control to ligand activity.

As extensively reviewed [137,226–228], upon binding of a TGF\(\beta\) superfamily member to its receptor, the formation of a heterodimeric serine/threonine kinase complex is induced, which is composed of a pair of high affinity type I and type II receptors (see Table 2 and Figure 3). In addition, there are a number of type III co-receptors, able to indirectly regulate signalling by binding to TGF\(\beta\) ligands and modulating their subsequent binding to type I and type II receptors. The type II serine/threonine kinases are constitutively active, and upon ligand binding undergo a conformational change, which leads to the recruitment and phosphorylation of the appropriate type I receptors. Type I receptors then specifically recognise and phosphorylate receptor-regulated Smad proteins (R-Smads). In this regard, there are two classes of R-Smads: the TGF\(\beta\) responsive Smads, which include Smad 2 and 3, and the BMP-responsive Smads that include Smad 1, 5, and 8. Upon activation, R-Smads form a complex with the common-partner Smad (Co-Smad; Smad 4), and translocate to the nucleus to regulate the transcription of a multitude of target genes. Inhibitory Smads (I-Smads; Smad6/7) can also inhibit the receptor activation of R-Smads by mediating the degradation of receptors and R-Smads. While Smad 7 inhibits all TGF\(\beta\) superfamily members, Smad 6 is more specific towards the BMP subfamily [229].

Aside from canonical Smad-mediated TGF\(\beta\) superfamily signalling, other signalling pathways, such as the mitogen activated-protein kinase (MAPK) pathways have also been implicated in the process (reviewed [230]). These non-Smad-mediated pathways can either be directly activated by TGF\(\beta\) ligands, or can modulate the activity of TGF\(\beta\)-induced Smad signalling, allowing for crosstalk and modification/fine-tuning of initial Smad-mediated signals.

Table 2. Components of the TGF\(\beta\) Smad-dependent canonical signalling pathway.

| Molecular Category | TGF\(\beta\) Sub-Family Pathway 1 | BMP Sub-Family Pathway |
|--------------------|-----------------------------------|------------------------|
| Ligands            | TGF\(\beta\)s, Activin-Like, BMP3, Nodal | BMP2/4/7/8/9/10/11, BMP3, GDF3, GDF5/1/3/5/6/7, MIS |
| Type II receptors  | TβRII, ActRIIIA, ActRIIB           | BMPRII, ActRIIA, ActRIIB |
| Type I receptors   | ALK4, TβRI (ALK5), ALK7            | ALK1/4, BMP1A (ALK1), BMP1B (ALK6) |
| R-Smad             | Smad2/3                           | Smad1/5/6/7 |
| Co-Smad            | Smad4                             | Smad4 |
| I-Smad             | Smad7                             | Smad7 |

1 Alternative protein names are listed in brackets. TβR, TGF\(\beta\) receptor; MIS, muellerian inhibiting substance; BMPR, BMP receptor; ActR, activin receptor; ALK, activin receptor-like kinase. Table adapted from [226].
that instead TGF-β differentiates, evaluating if MSCs may have differing effects on chondrogenesis than TGF-β, leading to the deposition of a matrix rich in PG and collagen II. However, TGF-β has been shown to lead to abnormalities of the chondrogenic transcription factor Sox9 and to the extensive investigation of the BMP subfamily of the TGF-β signalling.

4.1.1. TGF-β Subfamily

TGF-β is crucial for cartilage maintenance and integrity, as highlighted by mutations in these genes/proteins, which have been shown to lead to OA-like pathology [231,232]. Lack of TGF-β signalling is also reported in aged mice, and murine models of OA [233,234]. This has led to the majority of in vitro chondrogenic differentiation protocols including supplementation with either TGF-β1 or TGF-β3, of which TGF-β3 has been shown to support chondrogenesis more efficiently [36–41,59,75,128,133,165]. It has generally been considered that TGF-β is the only well-established inducer of chondrogenesis, leading to the deposition of a matrix rich in PG and collagen II. However, TGF-β-induced chondrogenic differentiation of MSCs is also clearly accompanied by the expression of unwanted hypertrophic markers such as collagen X and MMP13 [36,37,39,41,75,128,165]. In addition, the ectopic transplantation of TGF-β-differentiated MSCs into the subcutaneous pouches of severe combined immunodeficient (SCID) mice has been shown to result in matrix calcification and vascular invasion [38]. Human articular chondrocytes have also been shown to be directed towards hypertrophy when expanded in the presence of TGF-β1 [235]. Interestingly, it has been reported that MSCs differentiated in the presence of TGF-β1 had significantly less mineralisation than those cultured with TGF-β3 [236]. However, taken together, these drawbacks have indicated that further refinement of chondrogenic differentiation protocols are required; as such, researchers are now looking into the use of other/combinations of chondrogenic factors, which can induce the differentiation of MSCs into a permanent articular chondrocyte-like phenotype, and

Figure 3. Schematic overview of TGFβ signalling. Binding of a TGFβ/BMP ligand to specific cell surface receptors induces the formation of a heteromeric type II/type I receptor complex. This binding is further regulated by type III receptors/co-receptors. Upon ligand binding, constitutively active type II receptors activate type I receptors. This then leads to the phosphorylation of R-Smads (Smad 2/3 for the TGFβ subfamily; Smad1/5/8 for the BMP subfamily). R-Smads then form heterodimeric complexes with Smad 4 (Co-Smad) and translocate to the nucleus, where they regulate gene expression through interaction with transcription factors (TFs). I-Smads (Smad 6/7) inhibit receptor activation of R-Smads. Besides the canonical Smad signalling pathway, non-Smad pathways, such as the MAPK pathways have also been implicated in TGFβ signalling. Figure adapted from [227].
that can withstand hypertrophy. For example, it has been indicated that PTHrP may be able to suppress the hypertrophic effects of TGFβ treatment, without affecting the deposition of a cartilaginous matrix [39,153,208,209]. The use of a wide range of BMP subfamily members are also being increasingly investigated (see Section 4.1.2). Importantly, mutations which lead to elevated TGFβ activity have also been associated with increased bone mass and ossification [237,238], and TGFβ supplementation has been shown to stimulate osteophyte formation in the murine knee joint [239]. This therefore highlights that TGFβ activity is not limited to the articular cartilage tissue of synovial joints, and may well help explain why TGFβ stimulation also leads to chondrocyte hypertrophy and mineralisation (i.e., if MSCs are not first primed towards the articular cartilage pathway). The medium formulations (including growth factors) that are currently used for inducing chondrogenic differentiation of MSCs are potentially overly simplistic, since the formation of permanent articular cartilage is likely to be dependent upon the cross-talk from multiple signalling pathways [20,240]. Investigation into the effects of other chondrogenic factors has therefore become vital.

4.1.2. BMP Subfamily

The unsatisfactory results from in vitro chondrogenic protocols using TGFβ supplementation has led to the extensive investigation of the BMP subfamily of the TGFβ superfamily [153,183–186,241–243]. BMPs have been shown to play a number of essential roles in endochondral ossification, and are important for chondrocyte proliferation and differentiation, helping to maintain the expression of the chondrogenic transcription factor Sox9 [244,245]. Dual knockdown of BMP2 and BMP4 in mice has been shown to lead to abnormalities in chondrogenic condensations, and severe disorganisation of chondrocytes within the growth plate [246,247]. BMP7 is also thought to play a particularly important role in articular cartilage maintenance, with intra-articular injections of the ligand being shown to delay cartilage degradation in mice [248]. In vitro studies have demonstrated that BMPs (predominantly BMP2/4/6/7) can also induce the chondrogenic differentiation of MSCs, and that the use of BMPs in combination with TGFβ is more effective at inducing chondrogenesis than TGFβ treatment alone [183–186,241,242]; however, the majority of these protocols have also led to extensive hypertrophic differentiation. In contrast, Weiss et al. [153] have suggested that BMP2/4/6/7 and IGF1 were individually not sufficient to induce chondrogenesis of MSCs and that instead TGFβ was also required. Again, when these growth factors were used in combination with TGFβ, collagen X expression was still observed. Caron et al. [186] also looked at the effects of both BMP2 and BMP7 in combination with TGFβ3, and found that while BMP2 promoted chondrocyte hypertrophy, BMP7 inhibited this terminal differentiation. Handorf and Li [243], have looked at varying growth factor requirements throughout the differentiation protocol, evaluating if MSCs may have differing requirements depending on their stage of differentiation. While sequential administration of TGFβ1 and BMP7 did not enhance chondrogenesis to a greater extent than treatment with both growth factors at every feed, the hypertrophic phenotype was significantly reduced; but, while these results were promising, additional reductions in hypertrophy were still thought to be required. This therefore further highlights that examination of factors that can repress hypertrophy, or prime MSCs early on towards a phenotypically stable articular cartilage state, is of profound importance. The identification of such a factor, and its use in combination/sequence with other TGFβ and BMP treatments may be key to generating a permanent hyaline cartilage tissue.

4.1.2.1. GDF5

GDF5, also known as BMP14 or cartilage derived morphogenetic protein 1 (CDMP1), is a particularly interesting member of the BMP subfamily. It is synthesised as a 501 amino acid preprotein (UNIPROT accession number: P43026) [249]. Upon cleavage of the signal sequence (27 residues), the proregion (70 kDa) is proteolytically removed, leaving a 13.5 kDa (120 amino acid) monomer, which will then go on to form a disulfide linked homodimer, or a heterodimer with another TGFβ superfamily member [250–252]. GDF5 is well established as playing a critical role in joint development.
and maintenance [23,171–173,253–256]. Its importance was first highlighted in mice carrying the brachypodism (bp) mutation, which results in changes to the length and number of bones in the limbs, and was found to be the result of mutations in the GDF5 gene [253]; GDF5 was also shown to be highly expressed in the joint interzone, and that mice lacking both GDF5 and GDF6 have further spread joint defects and skeletal growth retardation [257,258]; highlighting its importance in the formation of synovial joints. Importantly, GDF5 has also been shown to be required for proper joint formation and homeostasis in humans, and is predominantly expressed in areas of cartilage formation during embryonic development [171,259–263]. Loss of function mutations in the human GDF5 gene have been shown to result in a number of chondrodysplasias such as Grebe and Hunter-Thompson syndromes [259,263], and a single nucleotide polymorphism in the 5′UTR of human GDF5 has also been linked to OA susceptibility [261]. In contrast, overexpression of GDF5 has been shown to enhance chondrogenesis, increase the length and width of bones, and lead to joint fusions [260,262]. Despite the clear importance of GDF5 for skeletal formation, its use for chondrogenic in vitro differentiation protocols and regeneration strategies has been somewhat under-researched compared to other TGFβ superfamily members. However, previous work comparing the effects of TGFβ1 and GDF5 in fetal hMSCs via histological staining, has demonstrated that while TGFβ1 was more stimulatory in terms of GAG production, the combination of both TGFβ1 and GDF5 was synergistic [264]. In contrast, Feng et al. [176] indicated that compared to TGFβ1 (10 ng/mL), GDF5 (100 ng/mL) had a much greater effect on the chondrogenic differentiation of adipose derived rat MSCs, although collagen X levels appeared similar in both TGFβ and GDF5 treated pellets. Interestingly, MSCs transfected with GDF5 and implanted into full thickness articular cartilage defects in the knee joints of rabbits have shown promising results; demonstrating superior repair of hyaline cartilage compared to MSC implantation alone [265]. In addition, most crucially, Zhang et al. [177], have reported that GDF5 (100 ng/mL) inhibited hBMMSCs from expressing collagen X, while promoting the deposition of a cartilage-like matrix. Although their results were not compared to the more commonly used TGFβ, this study has sparked interest in GDF5 as a target for cartilage TE/repair strategies.

This is exemplified by our own recent work, showing that GDF5 can induce the chondrogenic differentiation of hMSCs, while overcoming the hurdle of hypertrophy [266]. In contrast to TGFβ1, we found that GDF5 induced aggrecan and Sox9 expression (both markers associated with chondrogenesis and ECM production [267]), without increasing the expression of collagen X (the major marker of chondrocyte hypertrophy [268]); see Figure 4A–C [266]. Our data suggests that GDF5 could be used to generate a clinically useful cartilage matrix with a high PG content, while maintaining the chondrocytes in a mature articular cartilage phenotype. As well as building on the results of Zhang et al. [177], our research [266] also complements several other studies published over the past few years [175,179,181,269]. For example, work in an OA rat model has demonstrated that GDF5 is expressed in healthy pre-hypertrophic cartilage, but is not evident as OA develops [175]. A further study with human chondrocytes has also shown that GDF5 stimulation promoted the expression of aggrecan, while inhibiting collagen X expression [179]. Consistent with what we observed, Murphy et al. [181] demonstrated that collagen X expression was significantly increased in the presence of TGFβ1 but not GDF5, i.e., in 7-day hMSC-derived chondrocyte pellets. However, our results are in contrast to another recent study suggesting that GDF5 can promote the hypertrophy of hMSC-derived chondrocyte pellets [180]. This work, however, looked at the effect of GDF5 in combination with TGFβ3, and not in isolation. We have demonstrated that GDF5 alone does not increase collagen X expression, and that the combination of GDF5 and TGFβ1 is no more potent at inducing collagen X than TGFβ1 treatment alone [266].

Thus, overall, there is increasing evidence supporting the potential use of GDF5 in cartilage TE strategies, especially when this growth factor is supplied to hMSCs in the absence of TGFβ. Future studies to look at the expression of a wider repertoire of genes involved in chondrogenesis, as well as additional biochemical assays (for example to quantify PG content) would help to further determine the effects of GDF5 on the chondrogenic differentiation of hMSCs. A recent study in human umbilical cord
perivascular stem cell-derived chondrocyte pellets demonstrated that GDF5 enhanced proliferation, but had no effect on the expression of chondrogenic-related genes [270], therefore indicating that the effect of GDF5 may be specific to the source of stem/stromal cells.

Figure 4. GDF5 promotes the chondrogenic differentiation of hMSCs without inducing hypertrophy, and its activity is modulated by GAGs. GDF5 promotes the expression of aggrecan (A) and Sox 9 (B), both markers associated with chondrogenesis and ECM production, in hMSC-derived chondrogenic pellets, but importantly, does this without inducing collagen X expression; (C), a marker of chondrocyte hypertrophy. The removal of endogenous HS-SPGs (HS) from the cell surface (by using heparinase) is positively correlated with the reduced level of GDF5 able to bind to the cell surface; (D), Exogenous heparin, but not equivalent doses of HS, inhibit GDF5-induced chondrogenic differentiation of hMSCs as monitored by aggrecan expression (E), * p < 0.05, ** p < 0.01, *** p < 0.001 versus no addition control; ## p < 0.01, ### p < 0.001, comparing heparin and HS of same dose (see [266] for full experimental details).

Importantly, the supplementation of hMSCs with GDF5 rather than TGFβ1/3 may provide an effective way to achieve the aim of forming hyaline rather than hypertrophic chondrocytes from hMSCs, and strongly suggests that a transition to using GDF5 in hMSC-based cartilage engineering strategies could help to overcome this long-standing hurdle [266]. However, hMSC heterogeneity [271], along with the inability of being able to form a scalable tissue, need to be overcome if successful clinical implementation is to be achieved. A more robust quality control of cell preparations, that can better predict clinical outcomes, and/or allow for the purification of subpopulations of cells with improved chondrogenic potential, is therefore of the upmost importance (see [272]). The difficulties surrounding the use of hMSCs, has also meant that researchers are now looking into alternative solutions to cell therapy. Conventionally the strategy would be to deliver expanded hMSCs (undifferentiated or
differentiated) to the repair site, but recent work has led to the opinion that the beneficial effects of hMSCs (or other stem cells) for tissue regeneration are not only due to cell restoration (and engraftment), but can also be attributed to the trophic factors that hMSCs release (see reviews [273,274]). As a result, research is now being directed into the identification and delivery of paracrine factors to the injury site, which can then modulate the environment and evoke a repair response from the resident cells [275–279]. These cell free approaches to tissue regeneration are exciting; e.g., overcoming the issues of cell sourcing, expansion and differentiation, as well as the strict regulatory issues that surround cell therapy. However, they come with other challenges, including the effective and safe delivery and/or controlled release of the bioactive factors [277,280]. These issues, which are relevant to both cell-free and cell-based regeneration strategies, will be explored in further detail within the following sections.

5. Glycosaminoglycans

As well as the difficulties in identifying the correct growth factors (and combinations thereof) to target for cartilage TE/regeneration strategies, the inherent instability of these proteins has also hampered their potential use. Growth factors are known to be susceptible to proteolytic degradation, are rapidly cleared from the injury site, and demonstrate burst release pharmacokinetics [281–283]. Together these factors have largely meant that supraphysiological quantities are required to get anywhere near the desired outcome, resulting in economically unsustainable costs for clinical translation [284–287]. In addition, the safety of growth factors is still under debate due to the increasing number that are being identified as proto-oncogenic, and this worry is only heightened by the high doses that are currently required [288–291]. The use of carriers that can reduce the quantity of exogenous growth factors required, through their localisation, protection or enhancement, or indeed that could harness endogenously produced growth factors, would therefore be ideal. Research into the use of GAGs for these approaches is proving particularly promising, and presents an attractive therapeutic opportunity for TE strategies [163,195,292–295].

In animal cells there are an estimated $10^5$–$10^6$ copies of HS and chondroitin sulfate (CS) on the cell surface, which range from ~40–160 nm in length; these GAGs are present at mg/mL concentrations in the ECM, highlighting their abundance and importance. Moreover, solution structure studies of sulfated GAGs have indicated that the oligosaccharides are highly dynamic, with a high degree of conformational plasticity (although forming stiffened random coils), allowing them to interact with a diverse number of proteins, and adding to the complexity of the interactions [296]. All GAGs, with the exception of HA (and heparin, once it has been secreted by mast cells), exist as PGs by attaching to serine residues on core proteins [297]; alternatively, keratan sulfate (KS) can be N-linked. PGs are produced by virtually all mammalian cells and can be found on the cell surface, within the ECM, or stored in secretory granules [298]. PGs can include only one GAG chain (e.g., decorin), or can have over 100 GAG chains, as is the case for aggrecan, the most predominant PG in cartilage [299,300]. Aggrecan consists of around 100 CS and 30 KS GAG chains extending from a large protein core of approximately 250 kDa [301]. In addition, this PG forms large aggregates (size, 1–4 µm), with hundreds of aggrecan molecules non-covalently bound on a single HA chain, where the interaction is stabilised by cartilage link protein [302]. The biomechanical properties of cartilage are largely due to the high negative charge provided by associated GAG chains, and the large size of the biopolymer, which is held in place by collagen networks. These factors render the PG immobile and unable to redistribute itself, allowing the matrix to become water swollen (Gibbs-Donnan effect), resistant to deformation, and able to resist compressive force [303]. The highly sulfated GAG chains of aggrecan are also responsible for sequestering and modulating the accessibility of molecules, such as growth factors, which bind via positively charged amino acid residues [304]. Other PGs expressed in cartilage include glypicans, cell surface syndecans and the large HSPG perlecan [162,305–307]. Via its HS chains, perlecan has strong affinity for collagen VI, and can bind to fibronectin and laminin, via both its HS chains and core protein [308]. Through these interactions perlecan has been shown to play an important role in
The physiological and pathological functions of PGs are vast; e.g., helping to define tissue form, and dynamically modulate function and remodeling during health and disease [309,310]. The specific functions of GAGs/PGs include ligand binding/immobilisation/retention, promotion of ligand/receptor interactions, protection of ligands from proteolysis, and facilitation of cell-cell and cell-matrix interactions [156,311–320]; all of which can have a profound effect on cellular activity. The variable lengths and sulfation patterns of GAG chains on PGs, makes them the most structurally complex and information dense molecules found in nature [309]. Importantly, the intrinsic heterogeneity of sulfated GAGs allows for diverse patterning and multifunctionality, and as such the understanding of how these ‘sugar codes’ control tissue development and homeostasis is of huge therapeutic interest [321–324]. This is particularly apparent in the case of heparin/HS, where the huge degree of versatility has opened up the concept of ‘heparanomics’, seeking to better understand how HS structures interact with proteins to modulate biological activity [325,326]. It has been estimated that over $1 \times 10^6$ structurally different sequences are possible from a just a short HS octasaccharide; emphasising the point that these molecules could potentially accommodate an enormous number of protein-binding epitopes [327]. Research aimed at enabling a better understanding of this complexity and its functional consequences is ongoing [328–330], and it is ultimately hoped that this will allow for improved modulation of cellular activity and thus, TE/regeneration strategies.

Role of GAGs in Stem Cell Differentiation and Development

The extensive work of Merry and colleagues has highlighted the importance of HS and HSPGs in stem cell differentiation and embryonic development [331–335]. For example, the neural differentiation of mouse ESCs (mESCs) expressing green fluorescent protein (GFP) under the control of the neural marker Sox1, has been used to show that, compared to undifferentiated mESCs where HS sulfation was low, GFP-expressing neural progenitor cells demonstrated increased levels of both N- and O-sulfation, as supported by high levels of HS biosynthesis enzymes such as NDST4 and 3OST [331]. Variations in sulfation patterns between mESCs and neural progenitor cells were also confirmed using epitope specific antibodies, which show preferential binding to specific patterns of sulfation in the HS chain [331]. The group also demonstrated that the phage-display antibody RB4EA-12, which specifically binds to 6-O sulfated structures, had significantly increased expression in the Sox1-GFP expressing neural progenitors compared to the undifferentiated mESCs. Further to this, EXT1$^{-/-}$ mESCs (that are unable to form an HS polymer) placed in neural media were unable to differentiate, unless supplemented with soluble heparin. It was also shown that exogenously added GAGs, of particular sulfation patterns, concentrations, and chain lengths were also able to support the neural differentiation of wild type mESCs [334].

Taking a similar approach, the Merry group have also characterised changes in HS expression during differentiation of mESCs down the mesoderm/hematopoietic lineage, using a cell line where GFP is under the control of the Bry gene (which is expressed in mesoderm but not in ESCs), alongside a panel of HS sequence specific antibodies [332]. Of particular significance was the antibody HS4C3 which was specifically expressed within the emerging Bry$^+$ population, but that disappeared from the cell surface as cells differentiated into mature hematopoietic cells. In vivo findings also demonstrated that HS4C3 binding sequences were restricted to the mesoderm during gastrulation. The rapid turnover of the HS4C3 HS binding sequences at the cell surface during different stages of differentiation indicates how highly regulated and important HS sulfation patterns are during development, allowing different growth factors to bind at specific spatio-temporal locations to instigate specific signalling pathways. Understanding the details of HS sequences that are involved in the formation of various tissues, will therefore lead to a better understanding of how stem cell differentiation and development is regulated and controlled. In a subsequent study, EXT1$^{-/-}$ mESCs were only able to differentiate into hematopoietic lineages with the addition of heparin [333]. In contrast (and in contrast to the
previously discussed work in the neural differentiation system), the addition of chemically N- or O-desulfated heparin oligosaccharides, or heparin chains shorter than 18 saccharides, were unable to restore differentiation. Together these results highlight the widespread importance of HS in embryonic tissue development. While the low sulfation patterns seen in mESCs help to keep the cells in a pluripotent, undifferentiated state, dynamic changes in the sulfation patterns of HS appear to be required for growth factor activity and stem cell differentiation.

Consistent with the findings above, Nairn et al., [336] examined transcripts encoding the glycosylation machinery during stem cell differentiation, and demonstrated clear correlations between transcripts and changes in glycan structures. In addition, with more particular reference to the importance of GAGs in cartilage development, bioimaging has been used to demonstrate changes in the staining intensity of CS and HS during the chondrogenic differentiation of ESCs [337]. In addition, homozygous mice lacking either exostosin 1 or 2 (EXT1/EXT2; responsible for the polymerisation of growing HS chains), fail to gastrulate, while heterozygous mutants are likely to develop hereditary multiple exotoses, characterised by abnormal cartilage differentiation, premature hypertrophy and bony outgrowths [338,339]. In addition, Stickens et al. [339] showed that all EXT2+/− mice display abnormalities in cartilage differentiation, with disorganisation of chondrocytes and premature hypertrophy in the cartilage. Similarly, conditional knockdown of EXT1 in limb bud mesenchyme also leads to dysregulation of BMP signalling and delayed chondrogenic differentiation [340]. Furthermore, HS chains on the PG perlecan within the growth plate have been shown to be involved in the binding of FGF2 to its receptors FGFR1–4, leading to the regulation of chondrocyte proliferation and bone growth [162]. Importantly, however, perlecan can only deliver FGF2 to its receptors following the removal of CS; thus the CS chains on perlecan allow for the sequestration of FGF2 within the PCM of cartilage tissue, while the HS chains help with its delivery to receptors [161,162]. FGF2 has also been demonstrated to be liberated from perlecan HS chains upon injury or mechanical compression [341].

In humans, a mutation in 3′-phosphoadenosine 5′-phosphosulfate synthase 1 (PAPSS1; a bi-functional enzyme with both ATP sulfurylase and adenosine 5′-phosphosulfate kinase activity), which introduces a premature stop codon and disrupts GAG sulfation, as well as other mutations that lead to disruption in sulfation patterns, have been shown to result in osteochondrodysplasias (disorders in the development of cartilage/bone) [342]. A study has also shown that there is significantly higher expression of the endosulfatases, Sulf1 and Sulf2, in OA versus normal articular cartilage, indicating that changes in 6-O sulfation patterns post biosynthesis can contribute to cartilage pathology [343]. In addition, Sulf1 and Sulf2 have been shown to modulate cartilage homeostasis via their differential effects on BMP and FGF signalling [344]. Together these studies indicate that cartilage is particularly sensitive to sulfation patterns within GAG sequences, with key signalling pathways being dependent upon particular domain structures and sulfation patterns being present; see Table 1. The predominant role of GAGs and PGs in the formation and maintenance of the ECM of articular cartilage, makes them particularly interesting as factors for improving cartilage TE strategies, and a better understanding and identification of GAG sequences involved in articular cartilage formation and disease is clearly required.

Exogenously added HS, and heparin have been shown to stimulate cartilage nodule formation and growth in chick limb bud mesenchyme micromass cultures [345]. In contrast, although HS treatment alone does not appear to have an effect on the in vitro chondrogenic differentiation of MSCs, addition in combination with TGFβ3 and BMP2 has been shown to potentiate chondrogenic activity, more so than growth factor treatment alone [346,347]. It has been suggested that this may be due to exogenous HS limiting the ability of ligands to bind to endogenous HS PGs that would usually prevent/regulate their binding to receptors [346]. Consistent with this, overexpression of syndecan-3, a major HS PG expressed during chondrogenesis, was shown to impair the ability of BMP2 to promote chondrogenic differentiation [346]. On the other hand, the inclusion of the HS PG perlecan in biomaterials has been shown to enhance cartilage TE strategies, prolonging the release of BMP2 [348]. Exogenous HS has also been shown to prolong TGFβ1-mediated signalling in hMSCs [169]. In addition, a recent study has also
demonstrated that knockdown of perlecan inhibits the chondrogenic and adipogenic differentiation of SMSCs, but not osteogenic differentiation [349].

Ultimately, the ECM, and more specifically the PCM, which consists of the cell surface and immediate local ECM, plays a major role in maintaining homeostasis and directing cell behavior [350]. It provides a scaffold for cell attachment and spreading and helps provide the shape and mechanical properties of many tissues, including cartilage [304,351–354]. GAGs and PGs are key components of the PCM/ECM, keeping the matrix hydrated through their high negative charge, determining the compressive properties of the tissue, modulating morphogen gradients, and controlling the activity of ligands. A greater understanding of GAG structure within target tissues (the composition of which varies considerably with cell type and location), and how these are altered during development and disease, will therefore be of huge importance in the success of future TE strategies [355–357]. The elucidation of GAG structure/function relationships has lagged behind that of proteins and nucleic acids, largely because of limitations in the research methods available; e.g., GAGs cannot be amplified against a template, and as such cannot be sequenced in the same manner as DNA/RNA [297]. Adding to the challenge is their vast heterogeneity [321] and the high degree of sequence/conformational flexibility, likely underpinning the diversity and complexity of GAG-ligand interactions [296,328,358]. Despite these challenges, the persistence of the PG community has meant that much more advanced synthesis, modelling and sequencing tools are now becoming available (see [321,326,359–361]); in line with these developments, there is the hope that the full potential and full biological significance GAGs can soon be realised.

6. Biomaterials

A wide range of materials have been used for cartilage scaffold fabrication, including natural polymers such as collagen, fibrin and HA, as well as synthetic polymers such as polylactic acid (PLA) and polyglycolic acid (PGA), and self-assembling peptides (see [362]). Natural polymers have the obvious advantage of biological recognition, providing the cells with ECM molecules that can positively support cell function and adhesion [363–366]. Collagen, being a major component of the cartilage ECM, is a widely used natural polymer in cartilage TE, and is the most common scaffold to be incorporated into MACI procedures [55,60,367,368]. However, due to its abundance and versatility, collagen I (rather than type II) is usually used [369]. Collagen scaffolds have also been used in clinical trials immediately after microfracture treatment, although assessment of the benefit of this treatment still requires further analysis [370–372]. Multi-layered collagen scaffolds, which can more effectively mimic the zonal structure of articular cartilage, have also been recently examined in large animal models [373,374]. Histological analysis demonstrated that these scaffolds alone (i.e., cell-free) were able to initiate the formation of well-structured osteochondral tissue. Collagen I gels in combination with 2D micro patterning and single cell culture has also been used as a platform to investigate conditions that control chondrocyte de-differentiation [375]. This approach also offers a method from which niches for targeted differentiation of hMSCs could be investigated.

As well as collagen, HA and chitosan have also been extensively studied as natural polymers for cartilage TE/regeneration strategies. The HA-based scaffold, Hyalograft C, demonstrated promising results for the treatment of articular cartilage lesions, and like collagen, has also been used in MACI procedures [376,377]. However, results appeared less convincing for chronic lesions [378], and the product was recently discontinued failing EMA approval. More promisingly, a chitosan-based product, BST-CarGel, has been shown to result in better organised tissue repair compared to microfracture treatment alone [379], and further clinical evaluation will indeed be interesting to follow.

However, a number of concerns over the use of natural polymers, such as the feasibility of producing sufficient amounts of these materials for clinical applications, the degree of reproducibility, and assurance of pathogen removal, has led to a number of synthetic polymers being investigated as alternatives (as reviewed [380]). Synthetic polymers offer the benefits of processing flexibility and mass production, lack of immunological concerns, and greater control over degradation rates (see [381]).
Research involving synthetic polymers for cartilage TE is largely focused around degradable polyesters that have been FDA/EMA approved, such as PGA, PLA and their copolymer, poly(lactic-co-glycolic acid) (PLGA) (see [382,383]). PGA and PLA are degraded into glycolic acid and lactic acid respectively, both of which can be metabolised by the body and removed by natural pathways. A recent study has also indicated that the release of lactic acid from PLA scaffolds can be beneficial to chondrocyte ECM synthesis [384]. PLA contains a methyl side group within each monomer unit, which contributes to its hydrophobicity and slower degradation rate compared to PGA [385]. While the greater stability and high compressive strength of PLA may be beneficial to cartilage TE strategies [386], the hydrophobicity of the polymer limits cell attachment and viability [387]. In contrast to PLA, PGA is a rigid polymer with a high degree of crystallinity [385]; it is also hydrophilic and lacks methyl groups, which contributes to its higher degradation rate. The copolymer PLGA can therefore be used to tailor degradation rates and compressive strength, with altered ratios of PLA and PGA being used to fine-tune these properties. One study looked at the effects of PLGA composition on the cell adhesion and growth of bovine articular chondrocytes [388]; here non-woven PGA meshes were coated with varying quantities of PLA via solvent evaporation to give composites with PLA contents ranging from 0 to 68%. The compressive strength and degradation time was shown to increase with increasing PLA content, with those meshes containing 68% PLA lasting for 45 days with a compressive modulus of 20 kPa, while 0% PLA meshes degraded after just 5 days and had a compressive modulus of 1 kPa; however, the hydrophobic PLA was shown to decrease cell seeding efficiency, adhesion and proliferation. PLGA is also considered an expensive synthetic polymer and, as such, the relatively inexpensive polycaprolactone (PCL) is also being widely incorporated into many strategies [389–391]. This polymer has also been used to overcome the brittle properties of PLGA and its flexibility means that electrospun PCL nanofibrous sheets can be rolled or moulded over surfaces, e.g., of articular joints; making them excellent candidates for skeletal TE strategies [392]. However, like PLA, the downside of PCL lies in its hydrophobic nature and lack of cell surface recognition sites. Together the relative positive and negative attributes of synthetic polymers means that co-polymerisation of various combinations of both synthetic and natural polymers has become an active area of research [389,391,393–398].

6.1. Electrospun Scaffolds

As well as choice of polymer, scaffold form is also an important factor to consider. Electrospinning is a popular, simple, and cost-effective technique which allows for the production of both nano- and microfibrous polymer scaffolds (see [399]). Briefly, the technique involves dissolving a polymer in a suitable solvent and feeding it through a metal capillary with a high voltage applied, sending a jet of highly charged molecules in solution towards a collector of opposite charge [400]. As the jet travels, the solvent is evaporated, leaving a mesh of polymer fibers. The applied voltage, working distance, rotation rate, choice of solvent, polymer concentration, and flow rate, are all parameters that can be adjusted to generate scaffolds with different characteristics and fiber morphologies [401]. A number of groups have used electrospun scaffolds for cartilage TE purposes [398,402,403]. Sonomoto et al. [398] demonstrated that their PLGA electrospun nanofibrous scaffolds (a 50:50 ratio of PLA to PGA) could induce the differentiation of hMSCs into chondrocytes, without the addition of any cytokines. Wise et al. [402], have also compared orientated PCL electrospun scaffolds of approximately 500 nm and 3000 nm fiber diameter. The group demonstrated that hMSCs preferentially orientated along the direction of the fibers, and maintained their orientation during chondrogenic differentiation. The nanofibrous scaffolds (500 nm) were shown to enhance chondrogenic differentiation when compared to the microfibrous scaffolds (3000 nm), as indicated by type II collagen and aggrecan expression.

An issue of electrospinning is the inherently small pore sizes generated (typically < 10 μm), and the low thickness of fibers (typically ranging from 10 to 10,000 nm in diameter; limited by the slow rate of production), which together means that 2D membranes with poor cellular infiltration rather than true 3D scaffolds are often formed [404,405]. As such, techniques such as multilayering, or the incorporation
of hydrogels are being investigated [395,406–409]. Indeed, the native ECM consists of a fibrous protein network infiltrated with PGs, and so inclusion of a hydrogel within electrospun fibers seems a natural step in creating an ECM-mimetic with good levels of hydration, cell infiltration and the potential for controlled release of bioactive factors. A technique involving the combination of electrospinning and electrospraying allows for the rapid fabrication of fiber/hydrogel hybrid scaffolds with improved and more uniform cell infiltration [407,409]. Chen et al. [395] have also recently designed a porous 3D scaffold using electrospun gelatin/PLA nanofibers crosslinked with HA. Chondrocytes cultured in the scaffolds remained viable, and histological staining demonstrated that the construct could enhance the repair of cartilage in vivo. In an alternative approach Coburn et al. [410] have developed a fiber-hydrogel composite, using a novel electrospinning technique, whereby the fibers are spun onto a water coagulation bath (rather than a solid surface). This allows for the formation of a rapid 3D fiber mesh with porosity suitable for hydrogel and cell infusion by means of simple mixing, and which is injectable and allows for homogenous tissue growth. They demonstrated that the density of the fibers within the constructs had an effect on tissue formation, with higher (40% dry weight) fiber density leading to greater MSC proliferation, while lower (10% dry weight) fiber density led to the greatest amount of matrix production following chondrogenesis. However, most characterisation of chondrogenesis in this study seems to have been on fiber-hydrogel composites made from stacking to form a 3D multilayered scaffold, rather than those made by the novel electrospinning technique. Compared to hydrogels alone, the stacked composites showed increased ECM production and greater response to mechanical stimulation, which resulted in a drastic increase in tissue production and near native levels of ECM (although ECM composition was not examined). Owing to the structured composition of articular cartilage, stacked composites, where each layer could be engineered to release different combinations of bioactive factors, may be more effective compared to one homogenous matrix. However, it should be noted that Coburn et al. [410] did not look at any markers of chondrocyte hypertrophy within their study.

6.2. Hydrogels

Hydrogels, water swollen hydrophilic polymers that are able to retain large quantities of water, are also popular for cartilage TE strategies, as they allow the formation of an effective 3D environment, can be processed in relatively mild conditions, and can be delivered in a minimally invasive manner (see [411,412]). The high water content of these gels creates a permeable matrix that can effectively mimic the ‘softness’ of cartilage, allowing for easy nutrient transfer, and which can signal to cells through both mechanical and chemical signals [411]. Given these properties, a wide range of both natural and synthetic hydrogels have been investigated for cartilage TE.

Examples of natural hydrogels frequently used for cartilage TE include fibrin and alginate. Ho et al. [413], compared the chondrogenic differentiation of BMMSCs seeded into fibrin hydrogels or fibrin-alginate composites. MSCs encapsulated within fibrin hydrogels showed increased mesenchymal condensation compared to fibrin-alginate constructs. The fibrin hydrogel encapsulated cells also showed increased chondrogenic differentiation with an increase in expression of collagen type II and aggrecan, and unlike the fibrin-alginate gels, did not appear to undergo hypertrophy. Interestingly though, alginate sulfate hydrogels have been shown to support a chondrocyte phenotype, and by combining alginate sulfate with nanocellulose it is possible to create a printable bioink, which could therefore allow for the spatial deposition of hydrogels with micrometer precision [414].

Nguyen et al. [7], have used different combinations of both synthetic and natural biopolymers to create hydrogels that can direct MSCs to differentiate and form ECM that is characteristic of the superficial, middle, or deep zones of articular cartilage (see Figure 1). For example, the combination of CS and MMP-sensitive peptides incorporated into polyethylene glycol (PEG) hydrogels reportedly led to high levels of type II collagen, low levels of PG expression, and a low compressive modulus characteristic of the superficial zone. In contrast, PEG-HA hydrogels induced low collagen II and high PG levels leading to a high compressive modulus more characteristic to that of the deep zone. In a
further study they demonstrated that layer-by-layer organisation of these hydrogels to create a single 3D scaffold can stimulate MSCs to differentiate and form a multilayered ECM similar to that of native articular cartilage [8].

Although hydrogels clearly offer much promise, a common issue is the toxicity problems associated with their chemical crosslinking [415]. A number of groups have therefore been investigating self-assembling peptides, made up of alternating hydrophilic and hydrophobic amino acids, which spontaneously form nanofiber scaffold hydrogels in NaCl solutions [416,417]. These self-assembling peptide hydrogels have the advantages of traditional hydrogels, but also avoid the use of toxic cross-links, harmful degradation products, and undesired immunological responses, making them a very attractive option for TE strategies [416]. Zhou et al. have developed a novel self-assembling peptide hydrogel, which is based around the co-assembly of two aromatic peptide amphiphiles; i.e., a structural peptide, fluorenylmethoxycarbonyl-diphenylalanine (Fmoc-FF) and a functional Fmoc-coupled RGD peptide (arginine-glycine-aspartic acid) (Fmoc-RGD), which corresponds to the cell-binding domain of the common ECM component fibronectin [418]. These short di/tri-peptides were shown to lead to the formation of a simple, cost-effective and bioactive hydrogel, which could be formed at neutral pH and effectively support cell attachment and spreading [418,419]. In addition, the versatility of the hydrogel means that it could also be decorated with other bioactive peptides. Similarly, Saiani et al. [420] have formed simple FEFEFKFK self-assembling octapeptide gels, which can also allow for the addition of bioactive peptides. The gels have been shown to self-assemble into β-sheet structures which can promote the round morphology and ECM production of chondrocytes [421].

Another limitation of hydrogels is that although MSCs undergoing chondrogenesis appear to prefer the softer material of a hydrogel over fibrous scaffolds, hydrogels are limited in their mechanical properties and, thus, this may negatively affect their survival within a joint [410]. As such, the use of fiber/hydrogel composites (as discussed in Section 6.1) is perhaps more likely to succeed in cartilage TE applications. However, considerable variation in the stiffness of self-assembling peptide hydrogels can be achieved through modulating the assembly conditions [422].

6.3. GAG Incorporation and Application

As discussed above, synthetic polymers are becoming increasingly popular in TE strategies, due to their reproducibility, non-immunogenic properties, and ability for up-scaled production [381]. However, the bioinert properties of synthetic materials, means that they usually have to be enhanced with additives such as expensive growth factors and cytokines. A more cost effective method of promoting the biofunctionality of synthetic scaffolds is thought to be through the use of GAGs (as discussed in Section 5).

A number of groups have pioneered the incorporation of GAGs into synthetic polymers, in order to produce functionalised scaffolds that combine the advantages of both synthetic and natural polymeric materials [335,423,424]. The incorporation of GAGs into hydrogels has utilised both covalent and non-covalent conjugation, which allow for the tunable adjustment of matrix characteristics and release of bioactive factors [424]. Kim et al. [425] seeded MSCs into photocrosslinkable HA hydrogels and cultured them with varying levels and durations of TGFβ3. Results demonstrated that relatively short-term exposure (7 days) to a high level of TGFβ3 (100 ng/mL) was more effective at inducing and maintaining cartilage formation when compared to constant delivery of a lower dose (10 ng/mL) over a 9-week period. In agreement with this, Bhakta et al. [189], have reported that heparin containing hydrogels reduced the burst release of BMP2, and sustained its activity, however, it was the initial burst release of BMP2 that was found to be important for optimal bone formation. These results indicate that the controlled degradation of biphasic scaffolds might allow for both the initial release of a high dose of growth factor, followed by prolonged activity [426].

Compared to the incorporation of GAGs into hydrogels, their immobilisation onto surfaces such as electrospun fibrous scaffolds has proved a more difficult task. Typically GAGs are anchored via covalent immobilisation, however, this has been shown to restrict the conformation of the bound
GAGs and their functionality [395,427–429]; e.g., in some situations the oligosaccharides are required to be internalised with signalling receptors [430–432]. Mahoney et al. [433] have developed a method for the non-covalent immobilisation of heparin to surfaces, which involves the use of cold plasma polymerisation of allylamine (ppAm) and avoids the need for labelling or chemical modification of the GAGs. The ppAm coats the surface in positively charged amine groups (–NH\(_2\)) [434], thereby allowing for the subsequent immobilisation of negatively charged GAGs. Plasma polymerisation is an established technology involving a vessel containing the vapour of a monomer at low pressure and an energy source (see [435]). Generally, the lower the ratio of power to monomer input rate, the higher the retention of monomer functionality, and in addition substrate temperature and the location of the plasma discharge relative to the substrate have been shown to affect surface chemistry [436]. Heparin immobilised onto ppAm-coated microtiter plates was able to interact with the heparin-binding proteins tumour necrosis factor-stimulated gene-6 (TSG-6), complement factor H, and the chemokine interleukin 8, whereas no functional heparin was present on untreated plates [433]. The positive charge of the allylamine coating, and the fact that the binding of heparin to the surface was salt-strength dependent indicates that the binding is at least partly through an ionic interaction [433,437]. It was also demonstrated that the ppAm coating could be used to create heparin gradients, and as well as heparin, can also support the functional binding of a wide range of GAGs, including CS, HA, and DS, although differences existed in their protein-binding capabilities depending on the surface chemistry to which they were adsorbed [437,438]. Yang et al. [439] have also coated stainless steel with ppAm, in order to develop a heparinised surface with biological function. They found that the heparin-binding surface inhibited the ability of human umbilical artery smooth muscle cells to adhere and proliferate, while enhancing that of human umbilical vein endothelial cells. The heparinised surface was also shown to inhibit thrombosis and promote re-endothelialisation in vivo. Meade et al. [335] translated the cold plasma polymerisation technology onto 3D constructs, developing a novel electrospun scaffold functionalised with GAGs non-covalently attached to the fiber surface via a ppAm coating. Bound GAGs were shown to be biologically active, restoring the ability of EXT1\(^{-/-}\) (HS-deficient) mESCs to differentiate down the neural lineage. Use of these scaffolds to investigate how GAGs can control the chondrogenic differentiation of hMSCs would therefore be interesting.

Whilst the incorporation of GAGs into biomaterials for improved TE strategies is not new, most attempts thus far have relied upon the use of poorly defined, heterogeneous, commercially available, preparations [188–190,192,194,335,395,426,428,439–441]. Although these strategies have generally proven effective, it is difficult to decipher specific structure-function relationships, and there is the worry that off-target effects may lead to sub-optimal or even detrimental effects [190,442–446]. In addition, the use of natural polymers, generally isolated from animal tissues, is a concern for clinical translation, not only due to the high levels of structural complexity, but also due to potential contamination. As recently as 2008, batches of heparin containing oversulfated CS, caused allergic-type reactions in patients and led to over 100 deaths, highlighting the need for a more controllable method of production [447]; this requirement has also recently been underlined by the identification of significant batch variation in commercially available preparations of HS used in research [448]. In an effort to purify more defined HS preparations, Cool and colleagues have successfully employed a peptide affinity isolation method [163,169,193,195,295]; this technique is suitable for scale-up to meet clinical demand, and has already been shown to be effective for potentiating the bioactivity and bioavailability of a number of growth factors. Further to this, the advancement of GAG modelling, sequencing tools over the past decade (see [326,359–361]), has also meant that the feasibility of generating synthetic GAG mimetics is progressing rapidly (see [449]). Chemoenzymatic methods using bacterial glycosyltransferases and synthetic UDP monosaccharide donors can now allow for the rapid production of structurally defined HS oligosaccharides [450], where the expression of HS biosynthetic enzymes at high levels in \(E.\) coli is making the process amenable to scale-up [451]. In addition, a novel strategy, whereby the 6-hydroxyl group of synthetic UDP-GlcNAc or UDP-GlcNS nucleotides is substituted by an azido group, is facilitating the synthesis of GAG mimetics in excellent yields [452];
here the azido moiety is converted to a sulfate group, leading to the production of oligosaccharides
with N-sulfates at both the 2- and 6- positions, allowing for close mimicry of natural N-sulfated, 6-O
sulfated GlcN residues. Improved understanding of the substrate specificity of C5 epimerase activity
has also meant that single product IdoA2S-GlcNS residues can now be irreversibly synthesised (i.e.,
rather than a mixture of GlcA and IdoA residues) [453]. These improved methods have meant that
over 30 synthetic heparin oligosaccharides have now been generated [449]. Ultimately, our ability to
uncover structure/function relationships in heparin/HS oligosaccharides is evolving rapidly, and with
this, the incorporation of more defined and specific HS sequences into TE strategies, in a cost-effective
manner, has now become a realistic goal.

7. The Problems Associated with Heparin

The high level of sulfation (and thus negative charge) carried by the GAG heparin, means that it
is able to bind, stabilise, and modulate the activity of a wide range of growth factors (see [454]). In
addition, the low cost, easy availability, and status of heparin as an already FDA- and EMA-approved
product, has meant that researchers are harnessing its properties, preferentially over that of other GAGs
(such as HS) to improve growth factor delivery [188,189,192,194,440]. Gaining particular prominence
is the use of heparin-loaded delivery vehicles for skeletal TE, where biomaterials decorated with
heparin have been extensively studied both in vitro and in vivo, for their ability to reduce the dosing
requirements and improve the therapeutic potential of BMPs, such as BMP2 [188,189,194,455–460].
Most of these studies have shown very promising results, with heparin-loaded biomaterials improving
BMP2 delivery, release, and osteogenic potential. It should be noted, however, that some inhibitory
effects of heparin on TGFβ superfamily members (and other proteins) have been reported when high
doses of the GAG are used [461,462]. In addition, in some cases the use of heparin-loaded biomaterials
have proven ineffective in vivo [192] and the long-term side effects have not been tested. Adding to
this, it has been shown in a rat ectopic model, that while collagen sponges soaked with BMP2 and
heparin did result in more bone formation compared to sponges soaked with BMP2 alone, the use of
an HS variant, rather than heparin, improved bone formation even further [190]. On this basis we
hypothesised that while heparin may enhance the retention/activity of BMP2 (or other growth factors)
under certain conditions, its binding ‘promiscuity’ means that it may also inhibit other factors that
play a role in the repair process, leading to sub-optimal or even deleterious results [266].

In addition to the potential off-target effects from heparin-incorporated biomaterials, the long-term
use of heparin as an anticoagulant has also resulted in a number of adverse clinical effects
being reported, including thrombocytopenia, vascular reactions and osteoporosis [442–445]. Cool
and colleagues, recently showed that heparin has donor-dependent effects on the stemness and
multipotency of hMSCs, and alters a number of signalling pathways associated with hMSC growth
and differentiation [446]. This raises a concern regarding the long-term use of heparin in the clinic
and its suitability in skeletal (and other) TE strategies. Furthermore, the potential of GDF5 in cartilage
TE strategies (see Section 4.1.2.1 above) lead us to investigate the interaction between GDF5 and
heparin. We identified GDF5 as a novel heparin/HS-binding protein, and demonstrated that heparin
(but not equivalent doses of HS) has a strong and clear inhibitory effect on the biological activity of
GDF5, even at doses 10-fold lower than those that would be clinically administered (Figure 4E) [266];
e.g., for patients with venous thromboembolism, the dose of heparin is usually maintained at 0.3 to
0.7 U/mL [463], while 10 nM of heparin used in our study equates to around 0.03 to 0.04 U/mL. In
addition, the inhibitory effect of heparin was seen across multiple hMSC donors and in the skeletal cell
line ATDC5. Given the importance of GDF5 for skeletal development, our results might help explain
the reported increased risk of developing osteoporosis following long-term heparin treatment [442,444],
and the variable (and disappointing) results seen with heparin-loaded biomaterials for skeletal
repair [190,192]. As illustrated in Figure 5, these data add further caution to the widespread use
of unfractionated heparin, both in the clinic and research settings.
Importantly, the HS used in our study did not show the same inhibitory effect as heparin on GDF5 activity (Figure 4E), indicating that HS may be a more suitable, safe and effective alternative for incorporation into TE strategies and stem cell expansion/differentiation protocols. However, it is hard to generalise when HS is so heterogeneous/diverse, and additional studies with better-defined HS preparations will of course be necessary to investigate this further. It should also be noted, that the unfractionated HS preparation we used (from porcine intestinal mucosa) did not significantly promote the activity of GDF5 either. Again, given the sequence diversity of HS [309,328,330], we anticipate that it will be possible to identify HS variants that promote GDF5 activity while retaining other beneficial effects on chondrocyte function/phenotype [266]. Previous work by the Cool/Nurcombe group indicates that isolation of HS variants by their affinity for particular growth factors, enables the selection of saccharides markedly more potent at promoting growth factor activity compared to using unfractionated HS starting material, with its intrinsically high level of compositional heterogeneity [190,193,195,295]. Thus, there is the possibility to potentiate the activity of specific growth factors, to the same extent as heparin, while avoiding the adverse and off-target effects stemming from heparin’s pleiotropic nature. Indeed, our work has revealed that the HS3 variant (selected through its affinity for BMP2 and shown to promote BMP2 activity [193,195]), did not inhibit GDF5 in the same manner as heparin [266]. Therefore, by using this particular HS preparation, there is the possibility of promoting BMP2 activity (as with heparin) without affecting the activity of GDF5, which would also be present at the injury site and be involved in the repair process [259,261,464]. This further highlights the therapeutic utility of developing selective HS variants, rather than using heparin. Interestingly, given that HS3 has a higher level of sulfation compared to unfractionated HS [193], and carries an overall charge more similar to heparin, it might have been expected to also inhibit GDF5 activity. However, this was not the case [266] and suggests that charge density is not the dominant feature in the interaction between GDF5 and heparin/HS, and that GAG structure and sequence may also play a role.

The clear inhibitory effects of exogenously added heparin on GDF5 activity, also led us to look at whether there was a requirement for endogenous HSPGs for GDF5 binding and localisation [266]. Previous studies have shown that overexpression of Drosophila HSPGs (i.e., division abnormally delayed (Dally) and Dally-like protein (Dlp)), result in enhanced Drosophila Decapentaplegic (Dpp) (an ortholog of vertebrate BMP2/4) signalling in the wing [465]. However, in HSPG deficient mutants, Dpp signalling is reduced [466]. These results therefore suggest a positive role for HSPGs in BMP signalling, perhaps by acting as a co-receptor for BMPs (as with FGF signalling [467,468]), or by modulating the bioavailability of BMPs at the cell surface. In favour of the latter hypothesis, our data [266] demonstrated that pericellular HSPGs play a key role in localising GDF5 to the cell surface, with a positive correlation between HSPG and GDF5 concentration being observed (Figure 4D). This is in good agreement with results reported for BMP2 [190,469], and further emphasises the importance of HSPGs in regulating the activity of many TGFβ superfamily members. These results then led us to hypothesise that the inhibitory effects of heparin on GDF5-induced activity may be due to the exogenous heparin

**Figure 5.** Urging caution over the use of heparin-loaded biomaterials for TE strategies. Heparin has an inhibitory effect on the activity of GDF5. As such, the incorporation of heparin-loaded biomaterials into skeletal TE strategies may have an inhibitory effect on GDF5, which is found naturally at the repair site and may also be important to the repair process, leading to suboptimal or deleterious outcomes.
out-competing the binding of pericellular HSPGs to GDF5; i.e., preventing GDF5 from localising to the cell surface and binding to its receptors to initiate downstream signalling/activity [266]. In agreement with this hypothesis, we were able to show that heparin (but not equivalent doses of HS) inhibited both GDF5 association to the cell surface, and GDF5-induced downstream Smad 1/5/8 signalling. Interestingly, we have recently observed that despite the lack of HS on the surface of HSPG-deficient CHO cells, and the decreased binding of GDF5 to these cells (compared to WT CHO), over a one hour time period, GDF5 signalling appeared similar in both cell lines (B.I. Ayerst, V. Nurcombe, A.J. Day, C.L.R. Merry and S. Cool, unpublished observations). We therefore suggest that while HSPGs are important for capturing and improving the bioavailability of GDF5 at the cell surface, and perhaps protecting the growth factor from proteolytic degradation, unlike the FGF family of growth factors [467,468], HSPGs are not critical for the interaction between GDF5 and its receptor for downstream signalling.

Since the inhibitory effect of exogenous heparin on GDF5 activity cannot simply be explained by a lack of accumulation of GDF5 at the cell surface, we suggest that the high affinity of heparin for GDF5 may also more directly inhibit GDF5-receptor interaction [266]. In addition, heparin has also been shown to inhibit the activity of heparanases [470–472]. While treatment with heparanase has been shown to stimulate chondrogenesis in micromass cultures of mouse embryo limb mesenchymal cells, the heparanase antagonist SST0001 (a heparin molecule, modified so that it lacks anticoagulant activity) strongly inhibited chondrogenesis [473]. Heparanase was also found to be over expressed in chondrocytes from patients with hereditary multiple exostoses; a skeletal disorder resulting in the formation of benign cartilagenous tumours. In this sense, the inhibitory effect of heparin on GDF5-induced activity seen in our study may be three fold; firstly the high affinity of heparin for GDF5 may directly inhibit GDF5-receptor interactions; secondly, heparin may bind to GDF5, preventing it from associating with cell surface HS, and therefore limiting its bioavailability; thirdly heparin may inhibit the activity of heparanases, thereby preventing GDF5 from being released from cell surface HSPGs, which is required for its interaction with its receptors and the initiation of downstream signaling (Figure 6).

The pleiotropic nature and off-target effects of heparin render it, in our opinion, unsuitable for use in TE strategies [442–446]. Importantly, HS-based therapeutics may offer improved outcomes, with a more direct and targeted control of growth factor activity [357]. Indeed, a successful strategy for isolating HS fractions according to their affinity for specific growth factors has been developed [163,193,195,295]. The arrangement of sulfate groups within HS chains are thought to align with basic amino acids within the heparin-binding domain of target proteins [474]. As such, the synthesis of peptides based on heparin-binding domains of proteins, can allow for affinity-based HS purification and the isolation of variants with increased growth factor potency. The technology has already been used to isolate HS variants with increased affinity for BMP2, VEGF165, and FGF2, which have all proved more efficacious than unfractionated HS for bone healing, angiogenesis, and stem cell expansion, respectively [163,193,195,295].

However, although successful in the cases above, limitations and challenges still exist in the widespread translation of this affinity purification platform. The use of linear heparin-binding peptide sequences is simplistic and not representative of the majority of heparin-protein interactions [158,169,475–478]. Ultimately, a major obstacle for the use of HS as a therapeutic lies in its vast heterogeneity, including (large) variations in its domain structure, sulfation level/pattern, and chain length [479]. On top of this, simple/effective methodologies for the sequencing of HS (and other GAG) chains are not yet available; thus, making it difficult to determine and interpret structure-function relationships [321]. In addition, the degree of specificity involved in protein-HS interactions is still very much open for discussion [159,328,330,480–482], adding doubt over how precisely protein-HS interactions can be controlled in vivo. Another important factor is that binding affinity does not always directly equate to activity, and broad recognition/specificity does not necessarily indicate
insignificance [483,484]; further adding to the complexities of developing a standard and effective method for targeted HS therapeutics.

Figure 6. Proposed methods of regulation of GDF5 activity by cell surface HSPGs and exogenously added heparin/HS. GDF5 is captured by HSPGs and accumulates at the cell surface, where it is then available for prolonged receptor binding and activity (A). In the absence of HSPGs the accumulation of GDF5 at the cell surface does not occur; GDF5 is still able to bind to its receptors to initiate activity, but the free ligand may be more susceptible to proteolytic degradation, and as a result the duration of downstream signalling and activity may not be as prolonged as in the presence of HSPGs; (B). Exogenous HS is able to bind to GDF5, but the higher affinity of cell surface HSPGs and receptors for GDF5 binding, means that the interaction is only transient in nature; cell surface HSPGs and receptors out-compete exogenous HS for the GDF5 interaction and, as a result, similar levels of signalling are seen as in situation part A. However, if a specific high affinity HS is used (at a high enough concentration) then inhibition would be seen (C). The high affinity of heparin for GDF5 means that this GAG binds and out-competes cell-surface HSPGs and receptors for binding. Both the accumulation of GDF5 at the cell surface and downstream signalling is inhibited (D).

Early on, the discovery of the sequence required for antithrombin III (ATIII) binding indicated that protein-HS interactions may be governed by specific sequences encoded within the primary structure of HS [485]. ATIII requires a 3-O-sulfated pentasaccharide in order to bind HS; i.e., GlcNS/NAc(6S)-GlcA-GlcNS(3S±6S)-IdoA(±2S)-GlcNS(±6S). Removal of the 3-O sulfate reduces the pentasaccharide’s affinity for ATIII by 17,000 fold [486]. However, since this finding, very few specific binding sequences have been identified, perhaps with the exception of FGF2 [155,487], and it has been suggested that the specificity of protein-HS interactions instead exists on many different levels; with some interactions being more specific than others [484]. The importance of sulfation patterning also exists at a chain level as well as at an individual saccharide level, where the distribution of sulfated domains within an HS chain can dictate binding specificity as well as (or in some cases, in
preference over) the specific sulfation sequence within those domains [159,488,489]. In support of this, HS chains produced by biosynthetic mutant mice, lacking HS 2-O-sulfotransferase activity, were still able to support FGF2 activity despite lacking 2-O sulfation, apparently because they had compensatory increases in N- and 6-O sulfation that maintained the charge distribution along the HS chain [489]. While HS contains regions of both high and low sulfation, known as NS and NA domains, respectively, heparin can be seen as a hypersulfated version of HS, with virtually continuous sulfation along its chains, i.e., forming essentially one long NS domain [479,490]. The overall high level of sulfation in heparin may therefore mask selectivity, leading to its pleiotropic nature. It has been suggested that in the case of some heparin/HS-protein interactions little specificity exists, and that instead overall charge density is the predominant factor in determining binding [483,491].

It is therefore likely that the extent of HS-binding specificity varies among different proteins, and that overall charge density, sulfate patterning, and sequence specificity on HS chains can all play an important role. In addition, while electrostatic interactions between sulfate/carboxyl groups in the GAG chains and surface exposed positively charged arginine/lysine residues in the protein are undoubtedly important [492], evidence also suggests that non-ionic interactions, such as van der Waals forces, hydrogen bonding, and aromatic ring stacking, can also play a role [328,481,492–496]. Interestingly, a recent study has suggested that the presence of residues such as asparagine or glutamine can help to identify heparin/HS binding sites [496]; where these uncharged residues confer specificity on the interaction. If correct, this could revolutionise the identification of HS-protein binding sites, and thus facilitate the design of targeted HS-based therapeutics.

While experimental and modelling techniques are rapidly advancing, we are still only in the infancy of being able to fully characterise protein-GAG interactions. The high level of heterogeneity, conformational flexibility, and uncertainty over binding mechanisms has meant that it is hard to model and identify interactions in a high-throughput and effective manner. In addition, it appears that the level of specificity involved in protein-GAG interactions is not universal, and high affinity HS variants may not always lead to the desired outcome. As such, the development of targeted HS therapeutics may be a more complex task than first envisioned. However, the fact that a degree of specificity does seem to exist for certain proteins, means that the use of HS variants, rather than heparin, to more tightly control protein activity, is still an exciting and realistic prospect. The development of more advanced modelling and sequencing tools, along with the further refinement of biomaterials for the delivery and application of HS variants, will however first be required, before the full potential of HS glycotherapeutics can be realised.

8. Conclusions

The low cost, easy availability, high level of sulfation, and status of heparin as an already FDA- and EMA- approved product, has meant that it has largely been the GAG of choice for TE applications. While the use of heparin has generally proven effective, the long-term side effects of its incorporation into biomaterials has not been tested, and in some cases the use of heparin-loaded biomaterials have proven ineffective in vivo. While heparin may enhance the retention/activity of certain growth factors under certain conditions, its binding ‘promiscuity’ means that it may also inhibit other factors that play an important role in the repair process, leading to suboptimal or even deleterious effects. These concerns have been highlighted by our recent work, indicating that exogenous heparin has a strong inhibitory effect on the activity of growth differentiation factor 5 (GDF5), a growth factor which plays a critical role in cartilage/skeletal formation and homeostasis. This is particularly worrying, given the increasing incorporation of heparin into skeletal TE strategies. Overall, there is growing evidence cautioning the use of heparin both in the clinic and in TE applications, and emphasising the need to transition to using more specific GAGs (e.g., HS derivatives or synthetics), with better-defined structures and fewer off-target effects, if optimal therapy is to be achieved. Importantly, the advancement of GAG modelling, sequencing and synthesis tools over the past decade are starting to allow for this transition; enabling us to move away from the use of heterogenous undefined GAG
preparations, and opening up more advanced opportunities for the use of GAGs in a more controlled and defined manner.

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References

1. Bhosale, M.A.; Richardson, J.B. Articular cartilage: Structure, injuries and review of management. *Br. Med. Bull.* 2008, 87, 77–95. [CrossRef] [PubMed]
2. Hardingham, T.E. Fell-Muir lecture: Cartilage 2010—The known unknowns. *Int. J. Exp. Pathol.* 2010, 91, 203–209. [CrossRef] [PubMed]
3. Correa, D.; Lietman, S.A. Articular cartilage repair: Current needs, methods and research directions. *Semin. Cell Dev. Bio.* 2016, 62, 67–77. [CrossRef] [PubMed]
4. Goldring, M.B. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Ther. Adv. Musculoskelet. Dis.* 2012, 4, 269–285. [CrossRef] [PubMed]
5. Schinagl, R.M.; Gurskis, D.; Chen, A.C.; Sah, R.L. Depth-dependent confined compression modulus of full-thickness bovine articular cartilage. *J. Orthop. Res.* 1997, 15, 499–506. [CrossRef] [PubMed]
6. Seror, J.; Zhu, L.; Goldberg, R.; Day, A.J.; Klein, J. Supramolecular synergy in the boundary lubrication of synovial joints. *Nat. Commun.* 2015, 6, 6497. [CrossRef] [PubMed]
7. Nguyen, L.H.; Kudva, A.K.; Guckert, N.L.; Linse, K.D.; Roy, K. Unique biomaterial compositions direct bone marrow stem cells into specific chondrocytic phenotypes corresponding to the various zones of articular cartilage. *Biomaterials* 2011, 32, 1327–1338. [CrossRef] [PubMed]
8. Nguyen, L.H.; Kudva, A.K.; Saxena, N.S.; Roy, K. Engineering articular cartilage with spatially-varying matrix composition and mechanical properties from a single stem cell population using a multi-layered hydrogel. *Biomaterials* 2011, 32, 6946–6952. [CrossRef] [PubMed]
9. Boschetti, F.; Peretti, G.M. Tensile and compressive properties of healthy and osteoarthritic human articular cartilage. *Biorheology* 2008, 45, 337–344. [PubMed]
10. Yang, N.; Meng, Q.J. Circadian Clocks in Articular Cartilage and Bone: A Compass in the Sea of Matrices. *J. Biol. Rhythm.* 2016, 31, 415–427. [CrossRef] [PubMed]
11. Shen, G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. *Orthod. Craniofac. Res.* 2005, 8, 11–17. [CrossRef] [PubMed]
12. Decker, R.S.; Koyama, E.; Pacifici, M. Genesis and morphogenesis of limb synovial joints and articular cartilage. *Matrix Biol.* 2014, 39, 5–10. [CrossRef] [PubMed]
13. Mitrovic, D. Development of the diarthrodial joints in the rat embryo. *Am. J. Anat.* 1978, 151, 475–485. [CrossRef] [PubMed]
14. Hunziker, E.B. Growth plate structure and function. *Pathol. Immunopathol. Res.* 1988, 7, 9–13. [CrossRef] [PubMed]
15. Hall, B.K.; Miyake, T. The membranous skeleton: The role of cell condensations in vertebrate skeletogenesis. *Anat. Embryol. (Berl)* 1992, 186, 107–124. [CrossRef] [PubMed]
16. Kronenberg, H.M. Developmental regulation of the growth plate. *Nature* 2003, 423, 332–336. [CrossRef] [PubMed]
17. Long, F.; Ornitz, D.M. Development of the endochondral skeleton. *Cold Spring Harb. Perspect. Biol.* 2013, 5, a008334. [CrossRef] [PubMed]
18. Kozhemyakina, E.; Lasser, A.B.; Zelzer, E. A pathway to bone: Signaling molecules and transcription factors involved in chondrocyte development and maturation. *Development* 2015, 142, 817–831. [CrossRef] [PubMed]
19. Camarero-Espinosa, S.; Rothen-Rutishauser, B.; Foster, E.J.; Weder, C. Articular cartilage: From formation to tissue engineering. *Biomater. Sci.* 2016, 4, 734–767. [CrossRef] [PubMed]
20. Zhong, L.; Huang, X.; Karperien, M.; Post, J.N. The Regulatory Role of Signaling Crosstalk in Hypertrophy of MSCs and Human Articular Chondrocytes. *Int. J. Mol. Sci.* 2015, 16, 19225–19247. [CrossRef] [PubMed]

21. Iwamoto, M.; Ohta, Y.; Larmour, C.; Enomoto-Iwamoto, M. Toward regeneration of articular cartilage. *Birth Defects Res. C Embryo Today* 2013, 99, 192–202. [CrossRef] [PubMed]

22. Pacifici, M.; Koyama, E.; Shibukawa, Y.; Wu, C.; Tamamura, Y.; Enomoto-Iwamoto, M.; Iwamoto, M. Cellular and molecular mechanisms of synovial joint and articular cartilage formation. *Ann. N. Y. Acad. Sci.* 2006, 1068, 74–86. [CrossRef] [PubMed]

23. Iwamoto, M.; Tamamura, Y.; Koyama, E.; Komori, T.; Takeshita, N.; Williams, J.A.; Nakamura, T.; Enomoto-Iwamoto, M.; Pacifici, M. Transcription factor ERG and joint and articular cartilage formation during mouse limb and spine skeletogenesis. *Dev. Biol.* 2007, 305, 40–51. [CrossRef] [PubMed]

24. Koyama, E.; Shibukawa, Y.; Nagayama, M.; Sugito, H.; Young, B.; Yuasa, T.; Okabe, T.; Ochiai, T.; Kamiya, N.; Rountree, R.B.; et al. A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. *Dev. Biol.* 2008, 316, 62–73. [CrossRef] [PubMed]

25. Rountree, R.B.; Schoor, M.; Chen, H.; Marks, M.E.; Harley, V.; Mishina, Y.; Kingsley, D.M. BMP receptor signaling is required for postnatal maintenance of articular cartilage. *PLoS Biol.* 2004, 2, e355. [CrossRef] [PubMed]

26. Hyde, G.; Dover, S.; Aszodi, A.; Wallis, G.A.; Boot-Handford, R.P. Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms. *Dev. Biol.* 2007, 304, 825–833. [CrossRef] [PubMed]

27. Von der Mark, K.; Kirsch, T.; Nerlich, A.; Kuss, A.; Weseloh, G.; Glückert, K.; Stöss, H. Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. *Arthritis Rheum.* 1992, 35, 806–811. [CrossRef] [PubMed]

28. St-Jacques, B.; Hammerschmidt, M.; McMahon, A.P. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.* 1999, 13, 2072–2086. [CrossRef] [PubMed]

29. Yoshida, C.A.; Yamamoto, H.; Fujita, T.; Furuichi, T.; Ito, K.; Inoue, K.; Yamana, K.; Zanma, A.; Takada, K.; Ito, Y.; Komori, T. Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev.* 2004, 18, 952–963. [CrossRef] [PubMed]

30. Mueller, M.B.; Tuan, R.S. Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis Rheum.* 2008, 58, 1377–1388. [CrossRef] [PubMed]

31. Shimizu, E.; Selvamurugan, N.; Westendorf, J.J.; Olson, E.N.; Partridge, N.C. HDAC4 represses matrix metalloproteinase-13 transcription in osteoblastic cells, and parathyroid hormone controls this repression. *J. Biol. Chem.* 2010, 285, 9616–9626. [CrossRef] [PubMed]

32. Davoli, M.A.; Lamplugh, L.; Beauchemin, A.; Chan, K.; Mordier, S.; Mott, J.S.; Murphy, G.; Docherty, A.J.; Leblond, C.P.; Lee, E.R. Enzymes active in the areas undergoing cartilage resorption during the development of the secondary ossification center in the tibiae of rats aged 0–21 days: II. Two proteinases, gelatinase B and collagenase-3, are implicated in the lysis of collagen fibrils. *Dev. Dyn.* 2001, 222, 71–88. [PubMed]

33. Yang, L.; Tsang, K.Y.; Tang, H.C.; Chan, D.; Cheah, K.S. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. *Proc. Natl. Acad. Sci. USA* 2014, 111, 12097–12102. [CrossRef] [PubMed]

34. Drissi, H.; Zusckik, M.; Rosier, R.; O’Keefe, R. Transcriptional regulation of chondrocyte maturation: Potential involvement of transcription factors in OA pathogenesis. *Mol. Aspects Med.* 2005, 26, 169–179. [CrossRef] [PubMed]

35. Zhang, W.; Chen, J.; Zhang, S.; Ouyang, H.W. Inhibitory function of parathyroid hormone-related protein on chondrocyte hypertrophy: The implication for articular cartilage repair. *Arthritis Res. Ther.* 2012, 14, 221. [PubMed]

36. Johnstone, B.; Hering, T.M.; Caplan, A.I.; Goldberg, V.M.; Yoo, J.U. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* 1998, 238, 265–272. [CrossRef] [PubMed]

37. Yoo, J.U.; Barthel, T.S.; Nishimura, K.; Solchaña, L.; Caplan, A.I.; Goldberg, V.M.; Johnstone, B. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J. Bone Joint Surg. Am.* 1998, 80, 1745–1757. [CrossRef] [PubMed]
38. Pelttari, K.; Winter, A.; Steck, E.; Goetzke, K.; Henning, T.; Ochs, B.G.; Aigner, T.; Richter, W. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum.* 2006, 54, 3254–3266. [CrossRef] [PubMed]

39. Kafienah, W.; Mistry, S.; Dickinson, S.C.; Sims, T.J.; Learmonth, I.; Hollander, A.P. Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients. *Arthritis Rheum.* 2007, 56, 177–187. [CrossRef] [PubMed]

40. Kreuz, P.C.; Steinwachs, M.; Erggelet, C.; Krause, S.J.; Ossendorf, C.; Maier, D.; Ghanem, N.; Uhl, M.; Haag, M. Classification of graft hypertrophy after autologous chondrocyte implantation of full-thickness chondral defects in the knee. *Osteoarthr. Cartil.* 2007, 15, 1339–1347. [CrossRef] [PubMed]

41. Murdoch, A.D.; Grady, L.M.; Ablett, M.P.; Katopodi, T.; Meadows, R.S.; Hardingham, T.E. Chondrogenic differentiation of human bone marrow stem cells in transwell cultures: Generation of scaffold-free cartilage. *Stem. Cells* 2007, 25, 2786–2796. [PubMed]

42. Chen, S.; Fu, P.; Cong, R.; Wu, H.; Pei, M. Strategies to minimize hypertrophy in cartilage engineering and regeneration. *Genes Dis.* 2015, 2, 76–95. [CrossRef] [PubMed]

43. Hunziker, E.B.; Lippuner, K.; Keel, M.J.; Shintani, N. An educational review of cartilage repair: Precepts & practice-myths & misconceptions-progress & prospects. *Osteoarthr. Cartil.* 2015, 23, 334–350. [PubMed]

44. Guijak, F. Biomechanical factors in osteoarthritis. *Best Pract. Res. Clin. Rheumatol.* 2011, 25, 815–823. [CrossRef] [PubMed]

45. Antony, B.; Jones, G.; Jin, X.; Ding, C. Do early life factors affect the development of knee osteoarthritis in later life: A narrative review. *Arthritis Res. Ther.* 2016, 18, 202. [CrossRef] [PubMed]

46. Fosang, A.J.; Beier, F. Emerging Frontiers in cartilage and chondrocyte biology. *Best Pract. Res. Clin. Rheumatol.* 2011, 25, 751–766. [CrossRef] [PubMed]

47. Xie, F.; Kovic, B.; Jin, X.; He, X.; Wang, M.; Silvestre, C. Economic and Humanistic Burden of Osteoarthritis: A Systematic Review of Large Sample Studies. *Pharmacoconomics* 2016, 34, 1087–1100. [CrossRef] [PubMed]

48. *Osteoarthritis in General Practice*; Arthritis Research UK: Scotland, UK, 2013.

49. Cross, M.; Smith, E.; Hoy, D.; Rolte, S.; Ackerman, I.; Fransen, M.; Bridgett, L.; Williams, S.; Guillemin, F.; Hill, C.L.; et al. The global burden of hip and knee osteoarthritis: Estimates from the global burden of disease 2010 study. *Ann. Rheum. Dis.* 2014, 73, 1323–1330. [CrossRef] [PubMed]

50. Kulkarni, K.; Karssiiens, T.; Kumar, V.; Pandit, H. Obesity and osteoarthritis. *Maturitas* 2016, 89, 22–28. [CrossRef] [PubMed]

51. Harris, J.D.; Siston, R.A.; Pan, X.; Flanigan, D.C. Autologous chondrocyte implantation: A systematic review. *J. Bone Joint Surg. Am.* 2010, 92, 2220–2233. [CrossRef] [PubMed]

52. Rodriguez-Merchan, E.C. Regeneration of articular cartilage of the knee. *Rheumatol. Int.* 2013, 33, 837–845. [CrossRef] [PubMed]

53. Tibesku, C.O.; Suzwart, T.; Kleffner, T.O.; Schlegel, P.M.; Jahn, U.R.; Van Aken, H.; Fuchs, S. Hyaline cartilage degenerates after autologous osteochondral transplantation. *J. Orthop. Res.* 2004, 22, 1210–1214. [CrossRef] [PubMed]

54. Brittberg, M.; Lindahl, A.; Nilsson, A.; Ohlsson, C.; Isaksson, O.; Peterson, L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Engl. J. Med.* 1994, 331, 889–895. [CrossRef] [PubMed]

55. Marlovits, S.; Stiessnig, G.; Kutschka-Lissberg, F.; Resinger, C.; Aldrian, S.M.; Vecsei, V.; Trauttag, S. Early postoperative adherence of matrix-induced autologous chondrocyte implantation for the treatment of full-thickness cartilage defects of the femoral condyle. *Knee Surg. Sports Traumatol. Arthrosoc.* 2005, 13, 451–457. [CrossRef] [PubMed]

56. Gobbi, A.; Kon, E.; Berruto, M.; Francisco, R.; Filardo, G.; Marcacci, M. Patellofemoral full-thickness chondral defects treated with Hyalograft-C: A clinical, arthroscopic, and histologic review. *Am. J. Sports Med.* 2006, 34, 1763–1773. [CrossRef] [PubMed]

57. Wood, J.J.; Malek, M.A.; Frassica, F.J.; Polder, J.A.; Mohan, A.K.; Bloom, E.T.; Braun, M.M.; Cote, T.R. Autologous cultured chondrocytes: Adverse events reported to the United States Food and Drug Administration. *J. Bone Joint Surg. Am.* 2006, 88, 503–507. [CrossRef] [PubMed]

58. Benya, P.D.; Padilla, S.R.; Nimni, M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* 1978, 15, 1313–1321. [CrossRef]
59. Tew, S.R.; Murdoch, A.D.; Rauchenberg, R.P.; Hardingham, T.E. Cellular methods in cartilage research: Primary human chondrocytes in culture and chondrogenesis in human bone marrow stem cells. *Methods* 2008, 45, 2–9. [CrossRef] [PubMed]

60. Ebert, J.R.; Smith, A.; Fallon, M.; Butler, R.; Nairn, R.; Breidahl, W.; Wood, D.J. Incidence, degree, and development of graft hypertrophy 24 months after matrix-induced autologous chondrocyte implantation: Association with clinical outcomes. *Am. J. Sports Med.* 2015, 43, 2208–2215. [CrossRef] [PubMed]

61. Martin, J.A.; Buckwalter, J.A. Telomere erosion and senescence in human articular cartilage chondrocytes. *J. Gerontol. A Biol. Sci. Med. Sci.* 2001, 56, B172–B179. [CrossRef] [PubMed]

62. Kuszel, L.; Trzeciak, T.; Richter, M.; Czarny-Ratajczak, M. Osteoarthritis and telomere shortening. *J. Appl. Genet.* 2015, 56, 169–176. [CrossRef] [PubMed]

63. Martin, G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* 1981, 78, 7634–7638. [CrossRef] [PubMed]

64. Williams, R.L.; Hilton, D.J.; Pease, S.; Willson, T.A.; Stewart, C.L.; Gearing, D.P.; Wagner, E.F.; Metcalf, D.; Nicola, N.A.; Gough, N.M. Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 1988, 336, 684–687. [CrossRef] [PubMed]

65. Williams, J.T.; Southerland, S.S.; Souza, J.; Calcutt, A.F.; Cartledge, R.G. Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. *Am. Surg.* 1999, 65, 22–26. [PubMed]

66. Beane, O.S.; Darling, E.M. Isolation, characterization, and differentiation of stem cells for cartilage regeneration. *Ann. Biomed. Eng.* 2012, 40, 2079–2097. [CrossRef] [PubMed]

67. Okita, K.; Ichisaka, T.; Yamanaka, S. Generation of germine-competent induced pluripotent stem cells. *Nature* 2007, 448, 313–317. [CrossRef] [PubMed]

68. Shih, C.C.; Forman, S.J.; Chu, P.; Slovak, M. Human embryonic stem cells are prone to generate primitive, undifferentiated tumors in engrafted human fetal tissues in severe combined immunodeficient mice. *Stem. Cells Dev.* 2007, 16, 893–902. [CrossRef] [PubMed]

69. Swijnenburg, R.J.; Schreper, S.; Govaert, J.A.; Cao, F.; Ransohoff, K.; Sheikh, A.Y.; Haddad, M.; Connolly, A.J.; Simonetti, D.W.; Craig, S.; Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Stem Cells Dev.* 2010, 19, 143–147. [CrossRef] [PubMed]

70. Polo, J.M.; Liu, S.; Figueroa, M.E.; Kulalert, W.; Emini, S.; Tan, K.Y.; Apostolou, E.; Stadtfeld, M.; Li, Y.; Shioda, T.; et al. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Proc. Natl. Acad. Sci. USA* 2008, 105, 12991–12996. [CrossRef] [PubMed]

71. Gutierrez-Aranda, I.; Ramos-Mejia, V.; Bueno, C.; Munoz-Lopez, M.; Real, P.; Macia, A.; Sanchez, L.; Ligero, G.; Garcia-Parez, J.L.; Menendez, P. Human induced pluripotent stem cells develop teratoma more efficiently and faster than human embryonic stem cells regardless the site of injection. *Stem. Cells* 2010, 28, 1568–1570. [CrossRef] [PubMed]

72. Jiang, Y.; Jahagirdar, B.N.; Reinhardt, R.L.; Schwartz, R.E.; Keene, C.D.; Ortiz-Gonzalez, X.R.; Reyes, M.; Lenvik, T.; Lund, T.; Blackstad, M.; et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002, 418, 41–49. [CrossRef] [PubMed]

73. Ham, O.; Song, B.W.; Lee, S.Y.; Choi, E.; Cha, M.J.; Lee, C.Y.; Park, J.H.; Kim, I.K.; Chang, W.; Lim, S.; et al. The role of microRNA-23b in the differentiation of MSC into chondrocyte by targeting protein kinase A signaling. *Biomaterials* 2012, 33, 4500–4507. [CrossRef] [PubMed]
78. Monsel, A.; Zhu, Y.G.; Gennai, S.; Hao, Q.; Liu, J.; Lee, J.W. Cell-based therapy for acute organ injury: Preclinical evidence and ongoing clinical trials using mesenchymal stem cells. *Anesthesiology* 2014, 121, 1099–1121. [CrossRef] [PubMed]
79. Le Blanc, K.; Tammik, C.; Rosendahl, K.; Zetterberg, E.; Ringden, O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp. Hematol.* 2003, 31, 890–896. [CrossRef] [PubMed]
80. Chamberlain, G.; Fox, J.; Ashton, B.; Middleton, J. Concise review: Mesenchymal stem cells: Their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem. Cells.* 2007, 25, 2739–2749. [CrossRef] [PubMed]
81. Uccelli, A.; Moretta, L.; Pistoia, V. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* 2008, 8, 726–736. [CrossRef] [PubMed]
82. Atoui, R.; Chiu, R.C. Mesenchymal stromal cells as universal donor cells. *Exp. Opin. Biol. Ther.* 2012, 12, 1293–1297. [CrossRef] [PubMed]
83. Le Blanc, K.; Rasmusson, I.; Sundberg, B.; Gotherstrom, C.; Hassan, M.; Uzunel, M.; Ringden, O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004, 363, 1439–1441. [CrossRef]
84. Ra, J.C.; Kang, S.K.; Shin, I.; Park, H.G.; Joo, S.A.; Kim, J.G.; Kang, B.C.; Lee, Y.S.; Nakama, K.; Piao, M.; et al. Stem cell treatment for patients with active rheumatoid arthritis: Safety and efficacy. *Stem. Cells Dev.* 2013, 22, 3192–3202. [CrossRef] [PubMed]
85. Wang, L.; Wang, L.; Cong, X.; Liu, G.; Zhou, J.; Bai, B.; Li, Y.; Bai, W.; Li, M.; Ji, H.; et al. Human umbilical cord mesenchymal stem cell therapy for patients with active rheumatoid arthritis: Health and disease. *Stem Cells Dev.* 2013, 22, 3192–3202. [CrossRef] [PubMed]
86. Wang, L.; Zhang, H.; Guan, L.; Zhao, S.; Gu, Z.; Wei, H.; Gao, Z.; Wang, F.; Yang, N.; Luo, L.; et al. Mesenchymal stem cells provide prophylaxis against acute graft-versus-host disease following allogeneic hematopoietic stem cell transplantation: A meta-analysis of animal models. *Oncotarget* 2016, 7, 61764–61774. [PubMed]
87. Dennis, J.E.; Charbord, P. Origin and differentiation of human and murine stroma. *Stem. Cells* 2002, 20, 205–214. [CrossRef] [PubMed]
88. Wakitani, S.; Mitsuoka, T.; Nakamura, N.; Toritsuka, Y.; Nakamura, Y.; Horibe, S. Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: Two case reports. *Cell Trans.* 2004, 13, 595–600. [CrossRef]
89. Giannini, S.; Buda, R.; Battaglia, M.; Cavallo, M.; Ruffilli, A.; Ramponi, L.; Pagliazzi, G.; Vannini, F. One-step repair in talar osteochondral lesions: 4-year clinical results and t2-mapping capability in outcome prediction. *Am. J. Sports Med.* 2013, 41, 511–518. [CrossRef] [PubMed]
90. Skowronski, J.; Rutka, M. Osteochondral lesions of the knee reconstructed with mesenchymal stem cells—Results. *Ortop. Traumatol. Rehabil.* 2013, 15, 195–204. [CrossRef] [PubMed]
91. Vega, A.; Martin-Ferrero, M.A.; Del Canto, F.; Alberca, M.; Garcia, V.; Munar, A.; Orozco, L.; Soler, R.; Fuertes, J.; Huguet, M.; et al. Treatment of Kneee Osteoarthritis With Allogeneic Bone Marrow Mesenchymal Stem Cells: A Randomized Controlled Trial. *Transplantation* 2015, 99, 1681–1690. [CrossRef] [PubMed]
92. Soler, R.; Orozco, L.; Munar, A.; Huguet, M.; Lopez, R.; Vives, J.; Coll, R.; Codinach, M.; Garcia-Lopez, J. Final results of a phase I-II trial using ex vivo expanded autologous Mesenchymal Stromal Cells for the treatment of osteoarthritis of the knee confirming safety and suggesting cartilage regeneration. *Knee* 2016, 23, 647–654. [CrossRef] [PubMed]
93. Vodyanik, M.A.; Yu, J.; Zhang, X.; Tian, S.; Stewart, R.; Thomson, J.A.; Slukvin, I.I. A mesoderm-derived precursor for mesenchymal stem and endothelial cells. *Cell Stem. Cell* 2010, 7, 718–729. [CrossRef] [PubMed]
94. Alrefaei, G.I.; Ayuob, N.N.; Ali, S.S.; Al-Karim, S. Effects of maternal age on the expression of mesenchymal stem cell markers in the components of human umbilical cord. *Folia Histochem. Cytobiol.* 2015, 53, 259–271. [CrossRef] [PubMed]
95. Friedenstein, A.J.; Gorskaja, J.F.; Kulagina, N.N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* 1976, 4, 267–274. [PubMed]
96. Caplan, A.I. Mesenchymal stem cells. *J. Orthop. Res.* 1991, 9, 641–650. [CrossRef] [PubMed]
97. Zuk, P.A.; Zhu, M.; Ashjian, P.; De Ugarte, D.A.; Huang, J.I.; Mizuno, H.; Alfonso, Z.C.; Fraser, J.K.; Benhaim, P.; Hedrick, M.H. Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell 2002, 13, 4279–4295. [CrossRef] [PubMed]
98. De Bari, C.; Dell’Accio, F.; Tylzanowski, P.; Luyten, F.P. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum. 2001, 44, 1928–1942. [CrossRef]
99. Erices, A.; Conget, P.; Minguell, J.J. Mesenchymal progenitor cells in human umbilical cord blood. Br. J. Haematol. 2000, 109, 235–242. [CrossRef] [PubMed]
100. Arufe, M.C.; De la Fuente, A.; Fuentes-Boquete, I.; De Toro, F.J.; Blanco, F.J. Differentiation of synovial CD-105(+) human mesenchymal stem cells into chondrocyte-like cells through spheroid formation. J. Cell Biochem. 2009, 108, 145–155. [CrossRef] [PubMed]
101. Heo, J.S.; Choi, Y.; Kim, H.S.; Kim, H.O. Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. Int. J. Mol. Med. 2016, 37, 115–125. [PubMed]
102. Sakaguchi, Y.; Sekiya, I.; Yagishita, K.; Muneta, T. Comparison of human stem cells derived from various mesenchymal tissues: Superiority of synovium as a cell source. Arthritis Rheum. 2005, 52, 2521–2529. [CrossRef] [PubMed]
103. Caterson, E.J.; Nesti, L.J.; Danielson, K.G.; Tuan, R.S. Human marrow-derived mesenchymal progenitor cells in articular cartilage: Regenerative Medicine. Ann. Biomed. Eng. 2016, 44, 1325–1354. [CrossRef] [PubMed]
104. Koga, H.; Muneta, T.; Ju, Y.J.; Nagase, T.; Nimura, A.; Mochizuki, T.; Ichinose, S.; von der Mark, K.; Sekiya, I. Synovial stem cells are regionally specified according to local microenvironments after implantation for cartilage regeneration. Stem. Cells 2007, 25, 689–696. [CrossRef] [PubMed]
105. Fras, B.A.; Pei, M. Synovium-derived stem cells: A tissue-specific stem cell for cartilage engineering and regeneration. Tissue Eng. Part B 2012, 18, 301–311. [CrossRef] [PubMed]
106. Jones, B.A.; Pei, M. Synovium-derived stem cells: A tissue-specific stem cell for cartilage engineering and regeneration. Tissue Eng. Part B 2012, 18, 301–311. [CrossRef] [PubMed]
107. Perdisa, F.; Gostynska, N.; Roffi, A.; Filardo, G.; Marcarci, M.; Kon, E. Adipose-Derived Mesenchymal Stem Cells for the Treatment of Articular Cartilage: A Systematic Review on Preclinical and Clinical Evidence. Stem. Cells Int. 2015, 2015, 597652. [CrossRef] [PubMed]
108. Caterson, E.J.; Nesti, L.J.; Danielson, K.G.; Tuan, R.S. Human marrow-derived mesenchymal progenitor cells: Isolation, culture expansion, and analysis of differentiation. Mol. Biotechnol. 2002, 20, 245–256. [CrossRef]
109. Hung, S.C.; Chen, N.J.; Hsieh, S.L.; Li, H.; Ma, H.L.; Lo, W.H. Isolation and characterization of size-sieved stem cells from human bone marrow. Stem. Cells 2002, 20, 249–258. [CrossRef] [PubMed]
110. De Ugarte, D.A.; Alfonso, Z.; Zuk, P.A.; Elbarbary, A.; Zhu, M.; Ashjian, P.; Benhaim, P.; Hedrick, M.H.; Fraser, J.K. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. Immunol. Lett. 2003, 89, 267–270. [CrossRef]
111. Dominici, M.; Le Blanc, K.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.; Krause, D.; Djean, R.; Keating, A.; Prokop, D.; Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006, 8, 315–317. [CrossRef] [PubMed]
112. Xie, X.J.; Wang, J.A.; Cao, J.; Zhang, X. Differentiation of bone marrow mesenchymal stem cells induced by myocardial medium under hypoxic conditions. Acta Pharmacol. Sin. 2006, 27, 1153–1158. [CrossRef] [PubMed]
113. Wu, R.; Liu, G.; Bharadwaj, S.; Zhang, Y. Isolation and myogenic differentiation of mesenchymal stem cells for urologic tissue engineering. Methods Mol. Biol. 2013, 1001, 65–80. [PubMed]
114. Dugan, J.M.; Cartmell, S.H.; Gough, J.E. Uniaxial cyclic strain of human adipose-derived mesenchymal stem cells and C2C12 myoblasts in coculture. J. Tissue Eng. 2014, 5, 2041731414530138. [CrossRef] [PubMed]
115. Mu, M.W.; Zhao, Z.Y.; Li, C.G. Comparative study of neural differentiation of bone marrow mesenchymal stem cells by different induction methods. Genet. Mol. Res. 2015, 14, 14169–14176. [CrossRef] [PubMed]
116. Jones, E.A.; Kinsey, S.E.; English, A.; Jones, R.A.; Straszynski, L.; Meredith, D.M.; Markham, A.F.; Jack, A.; Emery, P.; McGonagle, D. Isolation and characterization of bone marrow multipotent mesenchymal progenitor cells. Arthritis Rheum. 2002, 46, 3349–3360. [CrossRef] [PubMed]
117. Kalutz, N.; Ringe, J.; Holzwarth, C.; Charbord, P.; Niemeyer, M.; Jacobs, V.R.; Peschel, C.; Haupl, T.; Oostendorp, R.A. Novel markers of chondrocytal stem cells defined by genome-wide gene expression analysis of stromal cells from different sources. Exp. Cell Res. 2010, 316, 2609–2617. [CrossRef] [PubMed]

118. Mendicino, M.; Bailey, A.M.; Vonnacott, K.; Puri, R.K.; Bauer, S.R. MSC-based product characterization for clinical trials: An FDA perspective. Cell Stem. Cell 2014, 14, 141–145. [CrossRef] [PubMed]

119. Erdmann, G.; Suchanek, M.; Horn, P.; Graf, F.; Volz, C.; Horn, T.; Zhang, X.; Wagner, W.; Ho, A.D.; Boutros, M. Functional fingerprinting of human mesenchymal stem cells using high-throughput RNAi screening. Genome Med. 2015, 7, 46. [CrossRef] [PubMed]

120. Samsonraj, R.M.; Rai, B.; Sathiyanathan, P.; Puan, K.J.; Rotzscheke, O.; Hui, J.H.; Raghunath, M.; Stanton, L.W.; Nurcombe, V.; Cool, S.M. Establishing criteria for human mesenchymal stem cell potency. Stem. Cells 2015, 33, 1878–1891. [CrossRef] [PubMed]

121. Sacchetti, B.; Funari, A.; Remoli, C.; Giannicola, G.; Kogler, G.; Liedtke, S.; Cossu, G.; Serafini, M.; Sampaolesi, M.; Tagliafico, E.; et al. No identical “Mesenchymal Stem Cells” at Different Times and Sites: Human Committed Progenitors of Distinct Origin and Differentiation Potential Are Incorporated as Adventitial Cells in Microvessels. Stem. Cell Reports 2016, 6, 897–913. [CrossRef] [PubMed]

122. Shahdadfar, A.; Fronsdal, K.; Haug, T.; Reinhold, F.P.; Brinchmann, J.E. In vitro expansion of human mesenchymal stem cells: Choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem. Cells 2005, 23, 1357–1366. [CrossRef] [PubMed]

123. Avanzini, M.A.; Bernardo, M.E.; Cometa, A.M.; Perotti, C.; Zaffaroni, N.; Novara, F.; Visai, L.; Moretta, A.; Del Fante, C.; Villa, R.; et al. Generation of mesenchymal stromal cells in the presence of platelet lysate: A phenotypic and functional comparison of umbilical cord blood- and bone marrow-derived progenitors. Haematologica 2009, 94, 1649–1660. [CrossRef] [PubMed]

124. Torensma, R.; Prins, H.J.; Schrama, E.; Verwiel, E.T.; Martens, A.C.; Roelofs, H.; Jansen, B.J. The impact of cell source, culture methodology, culture location, and individual donors on gene expression profiles of bone marrow-derived and adipose-derived stromal cells. Stem. Cells Dev. 2013, 22, 1086–1096. [CrossRef] [PubMed]

125. Steinert, A.F.; Ghivizzani, S.C.; Rethwilm, A.; Tuan, R.S.; Evans, C.H.; Noth, U. Major biological obstacles for persistent cell-based regeneration of articular cartilage. Arthritis Res. Ther. 2007, 9, 213. [CrossRef] [PubMed]

126. Wakitani, S.; Nawata, M.; Tensho, K.; Okabe, T.; Machida, H.; Ohgushi, H. Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: Three case reports involving nine defects in five knees. J. Tissue Eng. Regen. Med. 2007, 1, 74–79. [CrossRef] [PubMed]

127. Huey, D.J.; Hu, J.C.; Athanasiou, K.A. Unlike bone, cartilage regeneration remains elusive. Science 2012, 338, 917–921. [CrossRef] [PubMed]

128. Mackay, A.M.; Beck, S.C.; Murphy, J.M.; Barry, F.P.; Chichester, C.O.; Pittenger, M.F. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng. 1998, 4, 415–428. [CrossRef] [PubMed]

129. Matsumoto, Y.; Hayashi, Y.; Schliewe, C.R.; Ikeya, M.; Kim, H.; Nguyen, T.D.; Sami, S.; Baba, S.; Barruet, E.; Nasu, A.; et al. Induced pluripotent stem cells from patients with human fibrodysplasia ossificans progressiva show increased mineralization and cartilage formation. Orphanet J. Rare Dis. 2013, 8, 190. [CrossRef] [PubMed]

130. De Kroon, L.M.; Narcisi, R.; Blaney Davidson, E.N.; Cleary, M.A.; van Beuningen, H.M.; Koevoet, W.J.; van Osch, G.J.; van der Kraan, P.M. Aktivin Receptor-Like Kinase Receptors ALK5 and ALK1 Are Both Required for TGFbeta-Induced Chondrogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells. PLoS ONE 2015, 10, e0146124. [CrossRef] [PubMed]

131. Lollì, A.; Narcisi, R.; Lambertini, E.; Penolazzi, L.; Angelozzi, M.; Kops, N.; Gasparini, S.; van Osch, G.J.; Piva, R. Silencing of Antichondrogenic MicroRNA-221 in Human Mesenchymal Stem Cells Promotes Cartilage Repair In Vivo. Stem. Cells 2016, 34, 1801–1811. [CrossRef] [PubMed]

132. Lollì, A.; Narcisi, R.; Lambertini, E.; Penolazzi, L.; Angelozzi, M.; Kops, N.; Gasparini, S.; van Osch, G.J.; Piva, R. The effect of oxygen tension on human articular chondrocyte matrix synthesis: Integration of experimental and computational approaches. Biotechnol. Bioeng. 2014, 111, 1876–1885.

133. Murdoch, A.D.; Hardingham, T.E.; Eyer, D.R.; Fernandes, R.J. The development of a mature collagen network in cartilage from human bone marrow stem cells in Transwell culture. Matrix Biol. 2016, 50, 6–26. [CrossRef] [PubMed]
134. Van Beeningen, H.M.; Glansbeek, H.L.; van der Kraan, P.M.; van den Berg, W.B. Osteoarthritis-like changes in the murine knee joint resulting from intra-articular transforming growth factor-beta injections. Osteoarthr. Cartil. 2000, 8, 25–33. [CrossRef] [PubMed]

135. Farrell, E.; van der Jagt, O.P.; Koevoet, W.; Kops, N.; van Manen, C.J.; Hellingman, C.A.; Jahr, H.; O’Brien, F.J.; Verhaar, J.A.; Weina, H.; et al. Chondrogenic priming of human bone marrow stromal cells: A better route to bone repair? Tissue Eng. Part C 2009, 15, 285–295. [CrossRef] [PubMed]

136. Mueller, M.B.; Fischer, M.; Zellner, J.; Berner, A.; Dienstknecht, T.; Prantl, L.; Kujat, R.; Nerlich, M.; Tuan, R.S.; Angele, P. Hypertrophy in mesenchymal stem cell chondrogenesis: Effect of TGF-beta isoforms and chondrogenic conditioning. Cells Tissues Organs 2010, 192, 158–166. [CrossRef] [PubMed]

137. Bauge, C.; Girard, N.; Lhuissier, E.; Bazille, C.; Boumediene, K. Regulation and Role of TGFbeta Signaling Pathway in Aging and Osteoarthritis Joints. Aging Dis. 2014, 5, 394–405. [PubMed]

138. Kuroda, R.; Ishida, K.; Matsumoto, T.; Akisue, T.; Fujioka, H.; Mizuno, K.; Ohgushi, H.; Wakitani, S.; Kurosaka, M. Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells. Osteoarthr. Cartil. 2007, 15, 226–231. [CrossRef] [PubMed]

139. Orozco, L.; Munar, A.; Soler, R.; Alberca, M.; Soler, F.; Huguet, M.; Sentis, J.; Sanchez, A.; Garcia-Sancho, J. Treatment of knee osteoarthritis with autologous mesenchymal stem cells: Two-year follow-up results. Transplantation 2014, 97, e66–e68. [CrossRef] [PubMed]

140. Wolfstadt, J.I.; Cole, B.J.; Ogilvie-Harris, D.J.; Viswanathan, S.; Chahal, J. Current concepts: The role of mesenchymal stem cells in the management of knee osteoarthritis. Sports Health 2015, 7, 38–44. [CrossRef] [PubMed]

141. Koh, Y.G.; Kwon, O.R.; Kim, Y.S.; Choi, Y.J.; Tak, D.H. Adipose-Derived Mesenchymal Stem Cells With Microfracture Versus Microfracture Alone: 2-Year Follow-up of a Prospective Randomized Trial. Arthroscopy 2016, 32, 97–109. [CrossRef] [PubMed]

142. Pak, J.; Lee, J.H.; Park, K.S.; Jeong, B.C.; Lee, S.H. Regeneration of Cartilage in Human Knee Osteoarthritis with Autologous Adipose Tissue-Derived Stem Cells and Autologous Extracellular Matrix. Biore. Open Access 2016, 5, 192–200. [CrossRef] [PubMed]

143. Park, Y.B.; Ha, C.W.; Lee, C.H.; Yoon, Y.C.; Park, Y.G. Cartilage Regeneration in Osteoarthritic Patients by a Composite of Allogeneic Umbilical Cord Blood-Derived Mesenchymal Stem Cells and Hyaluronate Hydrogel: Results From a Clinical Trial for Safety and Proof-of-Concept With 7 Years of Extended Follow-Up. Stem. Cells Transl. Med. 2016, 6, 613–621. [CrossRef] [PubMed]

144. Ruiz, M.; Cosenza, S.; Maumus, M.; Jorgensen, C.; Noel, D. Therapeutic application of mesenchymal stem cells in osteoarthritis. Exp. Opin. Biol. Ther. 2016, 16, 33–42. [CrossRef] [PubMed]

145. Correa, D.; Somoza, R.A.; Lin, P.; Greenberg, S.; Rom, E.; Duesler, L.; Welter, J.F.; Yayon, A.; Caplan, A.I. Sequential exposure to fibroblast growth factors (FGF) 2, 9 and 18 enhances hMSC chondrogenic differentiation. Osteoarthr. Cartil. 2015, 23, 443–453. [CrossRef] [PubMed]
152. Hellingman, C.A.; Koever, W.; Kops, N.; Farrell, E.; Jahr, H.; Liu, W.; Baatenburg de Jong, R.J.; Frenz, D.A.; van Osch, G.J. Fibroblast growth factor receptors in vitro and in vivo chondrogenesis: Relating tissue engineering using adult mesenchymal stem cells to embryonic development. *Tissue Eng. Part A* 2010, 16, 545–556. [CrossRef] [PubMed]

153. Weiss, S.; Hennig, T.; Bock, R.; Steck, E.; Richter, W. Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. *J. Cell Physiol.* 2010, 223, 84–93. [CrossRef] [PubMed]

154. Bianchi, G.; Banfi, A.; Mastrogiacomo, M.; Notaro, R.; Luzzatto, L.; Cancedda, R.; Quarto, R. Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp. Cell Res.* 2003, 287, 98–105. [CrossRef]

155. Turnbull, J.E.; Fernig, D.G.; Ke, Y.; Wilkinson, M.C.; Gallagher, J.T. Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J. Biol. Chem.* 1992, 267, 10337–10341. [PubMed]

156. Lin, X.; Buff, E.M.; Perrimon, N.; Michelson, A.M. Heparan sulfate proteoglycans are essential for FGF growth factor-beta 1 activity by heparin and fucoidan. *Biochem. J.* 1994, 302, 4397–4402. [PubMed]

157. Wijesinghe, S.J.; Ling, L.; Murali, S.; Qing, Y.H.; Hinkley, S.F.; Carnachan, S.M.; Bell, T.J.; Swaminathan, K.; van Wijnen, A.J.; et al. Affinity Selection of FGF2-Binding Heparan Sulfates for Ex Vivo Expansion of Human Mesenchymal Stem Cells. *J. Cell Physiol.* 2012, 232, 566–575. [PubMed]

158. Vincent, T.L.; McLean, C.J.; Full, L.E.; Peston, D.; Saklatvala, J. FGF-2 is bound to perlecan in the pericellular matrix of articular cartilage, where it acts as a chondrocyte mechanotransducer. *Osteoarthr. Cartil.* 2007, 15, 752–763. [CrossRef] [PubMed]

159. Sekiya, I.; Vuoristo, J.T.; Larson, B.L.; Prockop, D.J. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. *Proc. Natl. Acad. Sci. USA* 2002, 99, 4397–4402. [PubMed]
171. Chang, S.C.; Hoang, B.; Thomas, J.T.; Vukicevic, S.; Luyten, F.P.; Ryba, N.J.; Kozak, C.A.; Reddi, A.H.; Moos, M., Jr. Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J. Biol. Chem. 1994, 269, 28227–28234. [PubMed]

172. Storm, E.E.; Kingsley, D.M. GDF5 coordinates bone and joint formation during digit development. Dev. Biol. 1999, 209, 11–27. [CrossRef] [PubMed]

173. Masuya, H.; Nishida, K.; Furuichi, T.; Toki, H.; Nishimura, G.; Kawabata, H.; Yokoyama, H.; Yoshida, A.; Tominaga, S.; Nagano, J.; et al. A novel dominant-negative mutation in Gdf5 generated by ENU mutagenesis impairs joint formation and causes osteoarthritis in mice. Hum. Mol. Genet 2007, 16, 2366–2375. [CrossRef] [PubMed]

174. Kwong, F.N.; Hoyland, J.A.; Evans, C.H.; Freemont, A.J. Regional and cellular localisation of BMPs and their inhibitors' expression in human fractures. Int. Orthop. 2009, 33, 281–288. [CrossRef] [PubMed]

175. García-Diego-Cazares, D.; Aguirre-Sanchez, H.I.; Abarca-Buis, R.F.; Kouri, J.B.; Velasquillo, C.; Ibarra, C. Regulation of alpha5 and alphaV Integrin Expression by GDF-5 and BMP-7 in Chondrocyte Differentiation and Osteoarthritis. PLoS ONE 2015, 10, e0127166. [CrossRef] [PubMed]

176. Feng, G.; Wan, Y.; Balian, G.; Laurencin, C.T.; Li, X. Adenovirus-mediated expression of growth and differentiation factor-5 promotes chondrogenesis of adipose stem cells. Growth Factors 2008, 26, 132–142. [CrossRef] [PubMed]

177. Zhang, B.; Yang, S.; Sun, Z.; Zhang, Y.; Xia, T.; Xu, W.; Ye, S. Human mesenchymal stem cells induced by growth differentiation factor 5: An improved self-assembly tissue engineering method for cartilage repair. Tissue Eng. Part C 2011, 17, 1189–1199. [CrossRef] [PubMed]

178. Pattenden, G.; Yueh, D.J.; Hu, J.C.; Athanasiou, K.A. TGF-beta1, GDF-5, and BMP-2 stimulation induces chondrogenesis in expanded human articular chondrocytes and marrow-derived stromal cells. Stem. Cells 2015, 33, 762–773. [CrossRef] [PubMed]

179. Murphy, M.K.; Huey, D.J.; Hu, J.C.; Athanasiou, K.A. TGF-beta1, GDF-5, and BMP-2 stimulation induces chondrogenesis in expanded human articular chondrocytes and marrow-derived stromal cells. Stem. Cells 2015, 33, 762–773. [CrossRef] [PubMed]

180. Gandhi, N.S.; Mancera, R.L. Prediction of heparin binding sites in bone morphogenetic proteins (BMPs). Biochim. Biophys. Acta 2012, 1824, 1374–1381. [CrossRef] [PubMed]

181. Indrawattana, N.; Chen, G.; Tadokoro, M.; Shann, L.H.; Ohgushi, H.; Tateishi, T.; Tanaka, J.; Bunyaratvej, A. Growth factor combination for chondrogenic induction from human mesenchymal stem cells. Biochem. Biophys. Res. Commun. 2004, 320, 914–919. [CrossRef] [PubMed]

182. Sekiya, I.; Larson, B.L.; Vuoristo, J.T.; Reger, R.L.; Prockop, D.J. Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res. 2005, 320, 269–276. [CrossRef] [PubMed]

183. Steinert, A.F.; Proffen, B.; Kunz, M.; Hendrich, C.; Ghivizzani, S.C.; Noth, U.; Rethwilm, A.; Eulert, J.; Evans, C.H. Hypertrophy is induced during the in vitro chondrogenic differentiation of human mesenchymal stem cells by bone morphogenetic protein-2 and bone morphogenetic protein-4 gene transfer. Arthritis Res. Ther. 2009, 11, R148. [CrossRef] [PubMed]

184. Caron, M.M.; Emans, P.J.; Cremers, A.; Surtel, D.A.; Coolson, M.M.; van Rhijn, L.W.; Welting, T.J. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. Osteoarthr. Cartil. 2013, 21, 604–613. [CrossRef] [PubMed]

185. Irie, A.; Habuchi, H.; Kimata, K.; Sanai, Y. Heparin sulfate is required for bone morphogenetic protein-7 signaling. Biochem. Biophys. Res. Commun. 2003, 308, 858–865. [CrossRef] [PubMed]
189. Bhakta, G.; Rai, B.; Lim, Z.X.; Hui, J.H.; Stein, G.S.; van Wijnen, A.J.; Nurcombe, V.; Prestwich, G.D.; Cool, S.M. Hyaluronic acid-based hydrogels functionalized with heparin that support controlled release of bioactive BMP-2. *Biomaterials* **2012**, *33*, 6113–6122. [CrossRef] [PubMed]

190. Bramono, D.S.; Murali, S.; Rai, B.; Ling, L.; Poh, W.T.; Lim, Z.X.; Stein, G.S.; Nurcombe, V.; van Wijnen, A.J.; Cool, S.M. Bone marrow-derived heparan sulfate potentiates the osteogenic activity of bone morphogenetic protein-2 (BMP-2). *Bone* **2012**, *50*, 954–964. [CrossRef] [PubMed]

191. Kraushaar, D.C.; Rai, S.; Condac, E.; Nairn, A.; Zhang, S.; Yamaguchi, Y.; Moremen, K.; Dalton, S.; Wang, L. Heparan sulfate facilitates FGF and BMP signaling to drive mesoderm differentiation of mouse embryonic stem cells. *J. Biol. Chem.* **2012**, *287*, 22691–22700. [CrossRef] [PubMed]

192. Koo, K.H.; Lee, J.M.; Ahn, J.M.; Kim, B.S.; La, W.G.; Kim, C.S.; Im, G.I. Controlled delivery of low-dose bone morphogenetic protein-2 using heparin-conjugated fibrin in the posterolateral lumbar fusion of rabbits. *Artif. Organs* **2013**, *37*, 487–494. [CrossRef] [PubMed]

193. Murali, S.; Rai, B.; Dombrowski, C.; Lee, J.L.; Lim, Z.X.; Bramono, D.S.; Ling, L.; Bell, T.; Hinkley, S.; Nathan, S.S.; et al. Affinity-selected heparan sulfate for bone repair. *Biomaterials* **2013**, *34*, 5594–5605. [CrossRef] [PubMed]

194. Hettiliarachi, M.H.; Miller, T.; Temenoff, J.S.; Guldborg, R.E.; McDevitt, T.C. Heparin microparticle effects on presentation and bioactivity of bone morphogenetic protein-2. *Biomaterials* **2014**, *35*, 7228–7238. [CrossRef] [PubMed]

195. Rai, B.; Chatterjea, A.; Lim, Z.X.; Tan, T.C.; Sawyer, A.A.; Hosaka, Y.Z.; Murali, S.; Lee, J.J.; Fenwick, S.A.; Hui, J.H.; et al. Repair of segmental ulna defects using a beta-TCP implant in combination with a heparan sulfate glycosaminoglycan variant. *Acta Biomater.* **2015**, *28*, 193–204. [CrossRef] [PubMed]

196. Church, V.; Nohno, T.; Linker, C.; Marcelle, C.; Francis-West, P. Wnt regulation of chondrocyte differentiation. *J. Cell Sci.* **2002**, *115*, 4809–4818. [CrossRef] [PubMed]

197. Bradley, E.W.; Drissi, M.H. WNT5A regulates chondrocyte differentiation through differential use of the CaN/NFAT and IKK/NF-kappaB pathways. *Mol. Endocrinol.* **2010**, *24*, 1581–1593. [CrossRef] [PubMed]

198. Narcisi, R.; Cleary, M.A.; Brama, P.A.; Hoogduijn, M.J.; Tuysuz, N.; ten Berge, D.; van Osch, G.J. Long-term effects of insulin-like growth factor I overexpression via recombinant adeno-associated vector gene transfer upon the biological activities and differentiation potential of human bone marrow-derived mesenchymal stem cells. *Stem. Cell Reports* **2015**, *4*, 459–472. [CrossRef] [PubMed]

199. Studer, D.; Millan, C.; Ozturk, E.; Maniura-Weber, K.; Zenobi-Wong, M. Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells. *Eur. Cell Mater.* **2012**, *24*, 118–135, discussion 135. [CrossRef] [PubMed]

200. Dhoot, G.K.; Gustafsson, M.K.; Ai, X.; Sun, W.; Standiford, D.M.; Emerson, C.P., Jr. Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. *Science* **2001**, *293*, 1663–1666. [CrossRef] [PubMed]

201. Ai, X.; Do, A.T.; Lozynska, O.; Kusche-Gullberg, M.; Lindahl, U.; Emerson, C.P., Jr. QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. *J. Cell Biol.* **2003**, *162*, 341–351. [CrossRef] [PubMed]

202. Song, H.H.; Shi, W.; Xiang, Y.Y.; Filmus, J. The loss of glypicanc-3 induces alterations in Wnt signaling. *J. Biol. Chem.* **2005**, *280*, 2116–2125. [CrossRef] [PubMed]

203. Liu, S.; Zhang, E.; Yang, M.; Lu, L. Overexpression of Wnt11 promotes chondrogenic differentiation of bone marrow-derived mesenchymal stem cells in synergy with TGF-beta. *Mol. Cell Biochem.* **2014**, *390*, 123–131. [CrossRef] [PubMed]

204. Enomoto-Iwamoto, M.; Kitagaki, J.; Koyama, E.; Tamamura, Y.; Wu, C.; Kanatani, N.; Koike, T.; Okada, H.; Komori, T.; Yoneda, T.; et al. The Wnt antagonist Frzb-1 regulates chondrocyte maturation and long bone development during limb skeletogenesis. *Dev. Biol.* **2002**, *251*, 142–156. [CrossRef] [PubMed]

205. Hartmann, C.; Tabin, C.J. Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. *Cell* **2001**, *104*, 341–351. [CrossRef]

206. Frisch, J.; Venkatesan, J.K.; Rey-Rico, A.; Schmitt, G.; Madry, H.; Cucchiariini, M. Influence of insulin-like growth factor I overexpression via recombinant adeno-associated vector gene transfer upon the biological activities and differentiation potential of human bone marrow-derived mesenchymal stem cells. *Stem. Cell Res. Ther.* **2014**, *5*, 103. [CrossRef] [PubMed]

207. Moller, A.V.; Jorgensen, S.P.; Chen, J.W.; Larnkjaer, A.; Ledet, T.; Flyvbjerg, A.; Frystyk, J. Glycosaminoglycans increase levels of free and bioactive IGF-I in vitro. *Eur. J. Endocrinol.* **2006**, *155*, 297–305. [CrossRef] [PubMed]
208. Kim, Y.J.; Kim, H.J.; Im, G.I. PTHrP promotes chondrogenesis and suppresses hypertrophy from both bone marrow-derived and adipose tissue-derived MSCs. Biochem. Biophys. Res. Commun. 2008, 373, 104–108. [CrossRef] [PubMed]

209. Lee, J.M.; Im, G.I. PTHrP isoforms have differing effect on chondrogenic differentiation and hypertrophy of mesenchymal stem cells. Biochem. Biophys. Res. Commun. 2012, 421, 819–824. [CrossRef] [PubMed]

210. Zak, B.M.; Crawford, B.E.; Esko, J.D. Hereditary multiple exostoses and heparan sulfate polymerization. Biochim. Biophys. Acta 2002, 1573, 346–355. [CrossRef]

211. Koziel, L.; Kunath, M.; Kelly, O.G.; Vortkamp, A. Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. Dev. Cell 2004, 6, 801–813. [CrossRef] [PubMed]

212. Newfeld, S.J.; Wisotzkey, R.G.; Kumar, S. Molecular evolution of a developmental pathway: Phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. Genetics 1999, 152, 783–795. [PubMed]

213. Moses, H.L.; Roberts, A.B.; Derynck, R. The Discovery and Early Days of TGF-beta: A Historical Perspective. Cold Spring Harb Perspect Biol. 2016, 8. [CrossRef] [PubMed]

214. Constam, D.B.; Robertson, E.J. Regulation of bone morphogenetic protein activity by pro domains and proprotein convertases. J. Cell Biol. 1999, 144, 139–149. [CrossRef] [PubMed]

215. Griffith, D.L.; Keck, P.C.; Sampath, T.K.; Rueger, D.C.; Carlson, W.D. Three-dimensional structure of the N domain of Smad7 is essential for specific inhibition of transforming growth factor-beta signaling. J. Cell Biol. 2004, 164, 355–364. [CrossRef] [PubMed]

216. Zhai, G.; Dore, J.; Rahman, P. TGF-beta signal transduction pathways and osteoarthritis. J. Biochem. 2009, 144, 139–149. [CrossRef] [PubMed]

217. Yu, Q.; Stamenkovic, I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta superfamily containing a novel pattern of cysteines. J. Biol. Chem. 1993, 268, 3444–3449. [PubMed]

218. Corradini, E.; Babitt, J.L.; Lin, H.Y. The RGM/DRAGON family of BMP co-receptors. J. Biol. Chem. 2004, 279, 171–181. [PubMed]

219. Barcellos-Hoff, M.H.; Dix, T.A. Redox-mediated activation of latent transforming growth factor-beta 1. Mol. Endocrinol. 1996, 10, 1077–1083. [PubMed]

220. Munger, J.S.; Harpel, J.G.; Gleizes, P.E.; Mazzieri, R.; Nunes, I.; Rifkin, D.B. Latent transforming growth factor-beta: Structural features and mechanisms of activation. Kidney Int. 1997, 51, 1376–1382. [CrossRef] [PubMed]

221. Yu, Q.; Stamenkovic, I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev. 2000, 14, 163–176. [PubMed]

222. Anderson, S.B.; Goldberg, A.L.; Whitman, M. Identification of a novel pool of extracellular pro-myostatin in skeletal muscle. J. Biol. Chem. 2008, 283, 7027–7035. [CrossRef] [PubMed]

223. Harrison, C.A.; Al-Musawi, S.L.; Walton, K.L. Prodomains regulate the synthesis, extracellular localisation and activity of TGF-beta superfamily ligands. Growth Factors 2011, 29, 174–186. [CrossRef] [PubMed]

224. Akhurst, R.J.; Hata, A. Targeting the TGFbeta signalling pathway in disease. Nat. Rev. Drug Discov. 2012, 11, 790–811. [CrossRef] [PubMed]

225. Weiss, A.; Attisano, L. The TGFbeta superfamily signaling pathway. Wiley Interdiscip. Rev. Dev. Biol. 2013, 2, 47–63. [CrossRef] [PubMed]

226. Zhai, G.; Dong, J.; Rahman, P. TGF-beta signal transduction pathways and osteoarthritis. Rheumatol. Int. 2015, 35, 1283–1292. [CrossRef] [PubMed]

227. Hanyu, A.; Ishidou, Y.; Ebisawa, T.; Shimanuki, T.; Imamura, T.; Miyazono, K. The N domain of Smad7 is essential for specific inhibition of transforming growth factor-beta signaling. J. Cell Biol. 2001, 155, 1017–1027. [CrossRef] [PubMed]

228. Weiss, A.; Attisano, L. The TGFbeta superfamily signaling pathway. Wiley Interdiscip. Rev. Dev. Biol. 2013, 2, 47–63. [CrossRef] [PubMed]

229. Hanyu, A.; Ishidou, Y.; Ebisawa, T.; Shimanuki, T.; Imamura, T.; Miyazono, K. The N domain of Smad7 is essential for specific inhibition of transforming growth factor-beta signaling. J. Cell Biol. 2001, 155, 1017–1027. [CrossRef] [PubMed]

230. Zhang, Y.E. Non-Smad pathways in TGF-beta signaling. Cell Res. 2009, 19, 128–139. [CrossRef] [PubMed]
231. Yamada, Y.; Miyauchi, A.; Goto, J.; Takagi, Y.; Okuzumi, H.; Kanematsu, M.; Hase, M.; Takai, H.; Harada, A.; Ikeda, K. Association of a polymorphism of the transforming growth factor-beta1 gene with genetic susceptibility to osteoporosis in postmenopausal Japanese women. *J. Bone Miner Res.* **1998**, *13*, 1569–1576. [CrossRef] [PubMed]

232. Kizawa, H.; Kou, I.; Iida, A.; Sudo, A.; Miyamoto, Y.; Fukuda, A.; Mabuchi, A.; Kotani, A.; Kawakami, A.; Yamamoto, S.; et al. An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis. *Nat. Genet.* **2005**, *37*, 138–144. [CrossRef] [PubMed]

233. Blaney Davidson, E.N.; Scharstuhl, A.; Vitters, E.L.; van der Kraan, P.M.; van den Berg, W.B. Reduced transforming growth factor-beta signaling in cartilage of old mice: Role in impaired repair capacity. *Arthritis Res. Ther.* **2005**, *7*, R1338–R1347. [CrossRef] [PubMed]

234. Blaney Davidson, E.N.; Vitters, E.L.; van der Kraan, P.M.; van den Berg, W.B. Expression of transforming growth factor-beta (TGFbeta) and the TGFbeta signalling molecule SMAD-2P in spontaneous and instability-induced osteoarthritis: Role in cartilage degradation, chondrogenesis and osteophyte formation. *Ann. Rheum. Dis.* **2006**, *65*, 1414–1421. [CrossRef] [PubMed]

235. Narcisi, R.; Quarto, R.; Ulivi, V.; Muraglia, A.; Molfetta, L.; Giannoni, P. TGF beta-1 administration during ex vivo expansion of human articular chondrocytes in a serum-free medium redirects the cell phenotype toward hypertrophy. *J. Cell Physiol.* **2012**, *227*, 3282–3290. [CrossRef] [PubMed]

236. Cals, F.L.; Hellingman, C.A.; Koevoet, W.; Baatenburg de Jong, R.J.; van Osch, G.J. Effects of transforming growth factor-beta subtypes on in vitro cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells. *J. Tissue Eng. Regen Med.* **2012**, *6*, 68–76. [CrossRef] [PubMed]

237. Campos-Xavier, B.; Saraiva, J.M.; Savarrirayan, R.; Verloes, A.; Feingold, J.; Faivre, L.; Munnick, A.; Le Merrer, M.; Cormier-Daire, V. Phenotypic variability at the TGF-beta1 locus in Camurati-Engelmann disease. *Hum. Genet.* **2001**, *109*, 653–668. [CrossRef] [PubMed]

238. Janssens, K.; ten Dijke, P.; Ralston, S.H.; Bergmann, C.; Van Hul, W. Transforming growth factor-beta 1 mutations in Camurati-Engelmann disease lead to increased signaling by altering either activation or secretion of the mutant protein. *J. Biol. Chem.* **2003**, *278*, 7718–7724. [CrossRef] [PubMed]

239. Van Beuninghen, H.M.; van der Kraan, P.M.; Arntz, O.J.; van den Berg, W.B. Transforming growth factor-beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. *Lab. Invest.* **1994**, *71*, 279–290. [PubMed]

240. Somoza, R.A.; Welter, J.F.; Correa, D.; Caplan, A.I. Chondrogenic differentiation of mesenchymal stem cells: Challenges and unfulfilled expectations. *Tissue Eng. Part B* **2014**, *20*, 596–608. [CrossRef] [PubMed]

241. Lee, P.T.; Li, W.J. Chondrogenesis of Embryonic Stem Cell-Derived Mesenchymal Stem Cells Induced by TGFbeta1 and BMP7 through Increased TGFbeta Receptor Expression and Endogenous TGFbeta1 Production. *J. Cell Biochem.* **2017**, *118*, 172–181. [CrossRef] [PubMed]

242. Shen, B.; Wei, A.; Whitaker, S.; Williams, L.A.; Tao, H.; Ma, D.D.; Diwan, A.D. The role of BMP-7 in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in vitro. *J. Cell Biochem.* **2010**, *109*, 406–416. [CrossRef] [PubMed]

243. Denker, A.E.; Haas, A.R.; Nicoll, S.B.; Tuan, R.S. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. *Differentiation* **1999**, *64*, 67–76. [CrossRef] [PubMed]

244. Kobayashi, T.; Lyons, K.M.; McMahon, A.P.; Kronenberg, H.M. BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 18023–18027. [CrossRef] [PubMed]

245. Bandyopadhyay, A.; Tsuii, K.; Cox, K.; Harfe, B.D.; Rosen, V.; Tabin, C.J. Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet.* **2006**, *2*, e216. [CrossRef] [PubMed]

246. Bian, Q.; Jia, K.; Liu, S.F.; Shu, B.; Liang, Q.Q.; Zhou, C.J.; Zhou, Q.; Wang, Y.J. Inhibitory effect of YQHYRJ recipe on osteoblast differentiation induced by BMP-2 in fibroblasts from posterior longitudinal ligament of mice. *Pharmazie* **2011**, *66*, 784–790. [PubMed]
248. Sekiya, I.; Tang, T.; Hayashi, M.; Morito, T.; Ju, Y.J.; Mochizuki, T.; Muneta, T. Periodic knee injections of BMP-7 delay cartilage degeneration induced by excessive running in rats. J. Orthop. Res. 2009, 27, 1088–1092. [CrossRef] [PubMed]

249. Hotten, G.; Neidhardt, H.; Jacobowsky, B.; Pohl, J. Cloning and expression of recombinant human growth/differentiation factor 5. Biochem. Biophys. Res. Commun. 1994, 204, 646–652. [CrossRef] [PubMed]

250. McDonald, N.Q.; Hendrickson, W.A. A structural superfamily of growth factors containing a cystine knot motif. Cell 1993, 73, 421–424. [CrossRef]

251. Luyten, F.P. Cartilage-derived morphogenetic protein-1. Int. J. Biochem. Cell Biol. 1997, 29, 1241–1244. [CrossRef]

252. Thieme, T.; Patzschke, R.; Job, F.; Liebold, J.; Seemann, P.; Lilie, H.; Balbach, J.; Schwarz, E. Biophysical and structural characterization of a folded core domain within the proregion of growth and differentiation factor-5. FEBs J. 2014, 281, 4866–2587. [CrossRef] [PubMed]

253. Storm, E.E.; Huynh, T.V.; Copeland, N.G.; Jenkins, N.A.; Kingsley, D.M.; Lee, S.J. Limb alterations in Ayerst, B.I.; Smith, R.A.; Nurcombe, V.; Day, A.J.; Merry, C.L.; Cool, S.M. Growth Differentiation Factor 5-Mediated Enhancement of Chondrocyte Phenotype Is Inhibited by Heparin: Implications for the Use of Heparin in the Clinic and in Tissue Engineering Applications. Tissue Eng. Part A 2017, 23, 275–292. [CrossRef] [PubMed]

254. Hotten, G.C.; Matsumoto, T.; Kimura, M.; Bechtold, R.F.; Kron, R.; Ohara, T.; Tanaka, H.; Satoh, Y.; Okazaki, M.; Shirai, T.; et al. Recombinant human growth/differentiation factor 5 stimulates mesenchyme aggregation and chondrogenesis responsible for the skeletal development of limbs. Growth Factors 1996, 13, 65–74. [CrossRef] [PubMed]

255. Buxton, P.; Edwards, C.; Archer, C.W.; Francis-West, P. Growth/differentiation factor-5 (GDF-5) and skeletal development. J. Bone Joint Surg. Am. 2001, 83-A (Suppl. 1), S23–S30. [CrossRef]

256. Schwartz, Y.; Viukov, S.; Krief, S.; Zelzer, E. Joint Development Involves a Continuous Influx of Gdf5-Positive Cells. Cell Rep. 2016, 15, 2577–2587. [CrossRef] [PubMed]

257. Storm, E.E.; Kingsley, D.M. Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. Development 1996, 122, 3969–3979. [PubMed]

258. Settle, S.H., Jr.; Rountree, R.B.; Sinha, A.; Thacker, A.; Higgins, K.; Kingsley, D.M. Multiple joint and skeletal patterning defects caused by single and double mutations in the mouse Gdf6 and Gdf5 genes. Dev. Biol. 2003, 254, 116–130. [CrossRef]

259. Thomas, J.T.; Kilipatruck, M.W.; Lin, K.; Erlacher, L.; Lembessis, P.; Costa, T.; Tsipouras, P.; Luyten, F.P. Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1. Nat. Genet. 1997, 17, 58–64. [CrossRef] [PubMed]

260. Dawson, K.; Seeman, P.; Sebald, E.; King, L.; Edwards, M.; Williams, J., 3rd; Mundlos, S.; Krakow, D. GDF5 is a second locus for multiple-synostosis syndrome. Am. J. Hum. Genet. 2006, 78, 708–712. [CrossRef] [PubMed]

261. Miyamoto, Y.; Mabuchi, A.; Shi, D.; Kubo, T.; Takatori, Y.; Saito, S.; Fujioka, M.; Sudo, A.; Uchida, A.; Yamamoto, S.; et al. A functional polymorphism in the 5′ UTR of GDF5 is associated with susceptibility to osteoarthritis. Nat. Genet. 2007, 39, 529–533. [CrossRef] [PubMed]

262. Degenkolbe, E.; Konig, J.; Zimmer, J.; Walther, M.; Reissner, C.; Nickel, J.; Ploeger, F.; Raspopovic, J.; Sharpe, J.; Dathe, K.; et al. A GDF5 point mutation strikes twice—Causing BDA1 and SYNS2. PLoS Genet. 2013, 9, e1003846. [CrossRef] [PubMed]

263. Martinez-Garcia, M.; Garcia-Canto, E.; Fenollar-Cortes, M.; Aytes, A.P.; Trujillo-Tiebas, M.J. Characterization of an acromesomelic dysplasia, Grebe type case: Novel mutation affecting the recognition motif at the processing site of GDF5. J. Bone Miner Metab. 2016, 34, 599–603. [CrossRef] [PubMed]

264. Bai, X.; Xiao, Z.; Pan, Y.; Hu, J.; Pohl, J.; Wen, J.; Li. Cartilage-derived morphogenetic protein-1 promotes the differentiation of mesenchymal stem cells into chondrocytes. Biochem. Biophys. Res. Commun. 2004, 325, 453–460. [CrossRef] [PubMed]

265. Katayama, R.; Wakitani, S.; Tsumaki, N.; Morita, Y.; Matsushita, I.; Gejo, R.; Kimura, T. Repair of articular cartilage defects in rabbits using CDMP1 gene-transfected autologous mesenchymal cells derived from bone marrow. Rheumatology (Oxford) 2004, 43, 980–985. [CrossRef] [PubMed]

266. Ayerst, B.I.; Smith, R.A.; Nurcombe, V.; Day, A.J.; Merry, C.L.; Cool, S.M. Growth Differentiation Factor 5-Mediated Enhancement of Chondrocyte Phenotype Is Inhibited by Heparin: Implications for the Use of Heparin in the Clinic and in Tissue Engineering Applications. Tissue Eng. Part A 2017, 23, 275–292. [CrossRef] [PubMed]
267. DeLise, A.M.; Fischer, L.; Tuan, R.S. Cellular interactions and signaling in cartilage development. Osteoarthr. Cartil. 2000, 8, 309–334. [CrossRef] [PubMed]

268. Zheng, Q.; Zhou, G.; Morello, R.; Chen, Y.; Garcia-Rojas, X.; Lee, B. Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. J. Cell Biol. 2003, 162, 833–842. [CrossRef] [PubMed]

269. Tian, H.T.; Zhang, B.; Tian, Q.; Liu, Y.; Yang, S.H.; Shao, Z.W. Construction of self-assembled cartilage tissue from bone marrow mesenchymal stem cells induced by hypoxia combined with GDF-5. J. Huazhong Univ. Sci. Technolog. Med. Sci. 2013, 33, 700–706. [CrossRef] [PubMed]

270. An, B.; Heo, H.-R.; Lee, S.; Park, J.-A.; Kim, K.-S.; Yang, J.; Hong, S.-H. Supplementation of Growth Differentiation Factor-5 Increases Proliferation and Size of Chondrogenic Pellets of Human Umbilical Cord-Derived Perivascular Stem Cells. Tissue Eng. Regen. Med. 2015, 12, 181–187. [CrossRef]

271. Muraglia, A.; Cancedda, R.; Quarto, R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J. Cell Sci. 2000, 113, 1161–1166. [PubMed]

272. Roelofs, A.J.; Rocke, J.P.; de Bari, C. Cell-based approaches to joint surface repair: A research perspective. Osteoarthr. Cartil. 2013, 21, 892–900. [CrossRef] [PubMed]

273. Baraniak, P.R.; McDevitt, T.C. Stem cell paracrine actions and tissue regeneration. Regen. Med. 2010, 5, 121–143. [CrossRef] [PubMed]

274. Anthony, D.F.; Shiel, P.G. Exploiting paracrine mechanisms of tissue regeneration to repair damaged organs. Transpl. Res. 2013, 2, 10. [CrossRef] [PubMed]

275. Gnecchi, M.; Melo, L.G. Bone marrow-derived mesenchymal stem cells: Isolation, expansion, characterization, viral transduction, and production of conditioned medium. Methods Mol. Biol. 2009, 482, 281–294. [PubMed]

276. Lee, R.H.; Pulin, A.A.; Geo, M.J.; Kota, D.J.; Lystalo, J.; Larson, B.L.; Sempun-Prieto, L.; Delafontaine, P.; Prokop, D.J. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem. Cell 2009, 5, 54–63. [CrossRef] [PubMed]

277. Ko, I.K.; Lee, S.J.; Atala, A.; Yoo, J.J. In situ tissue regeneration through host stem cell recruitment. Exp. Mol. Med. 2013, 45, e57. [CrossRef] [PubMed]

278. Linero, I.; Chaparro, O. Paracrine effect of mesenchymal stem cells derived from human adipose tissue in bone regeneration. Plos ONE 2014, 9, e107001. [CrossRef] [PubMed]

279. Hacker, S.; Mittermayr, R.; Nickl, S.; Haider, T.; Leberherz-Eichinger, D.; Beer, L.; Mitterbauer, A.; Leiss, H.; Zimmermann, M.; Schweiger, T.; et al. Paracrine Factors from Irradiated Peripheral Blood Mononuclear Cells Improve Skin Regeneration and Angiogenesis in a Porcine Burn Model. Sci. Rep. 2016, 6, 25168. [CrossRef] [PubMed]

280. Martino, M.M.; Briquez, P.S.; Maruyama, K.; Hubbell, J.A. Extracellular matrix-inspired growth factor delivery systems for bone regeneration. Adv. Drug Deliv. Rev. 2015, 94, 41–52. [CrossRef] [PubMed]

281. Manning, M.C.; Patel, K.; Borchardt, R.T. Stability of protein pharmaceuticals. Pharm. Res. 1989, 6, 903–918. [CrossRef] [PubMed]

282. Lauer, G.; Sollberg, S.; Cole, M.; Flamme, I.; Sturzebecher, J.; Mann, K.; Krieg, T.; Eming, S.A. Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. J. Investig. Dermatol. 2000, 115, 12–18. [CrossRef] [PubMed]

283. Eppler, S.M.; Combs, D.L.; Henry, T.D.; Lopez, J.J.; Ellis, S.G.; Yi, J.H.; Annex, B.H.; McCluskey, E.R.; ZIoncheck, T.F. A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clin. Pharmacol. Ther. 2002, 72, 20–32. [CrossRef] [PubMed]

284. Simons, M.; Annex, B.H.; Laham, R.J.; Kleiman, N.; Henry, T.; Dauerman, H.; Udelson, J.E.; Gervino, E.V.; Pike, M.; Whitehouse, M.J.; et al. Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: Double-blind, randomized, controlled clinical trial. Circulation 2002, 105, 788–793. [CrossRef] [PubMed]

285. Shields, L.B.; Raque, G.H.; Glassman, S.D.; Campbell, M.; Vitaz, H.; Harpring, J.; Shields, C.B. Adverse effects associated with high-dose recombinant human bone morphogenetic protein-2 use in anterior cervical spine fusion. Spine 2006, 31, 542–547. [CrossRef] [PubMed]

286. Epstein, N.E. Complications due to the use of BMP/INFUSE in spine surgery: The evidence continues to mount. Surg. Neurol. Int. 2013, 4 (Suppl. 5), S343–S352. [CrossRef] [PubMed]
287. Sreekumar, V.; Aspera-Werz, R.H.; Tendulkar, G.; Reumann, M.K.; Freude, T.; Breitkopf-Heinlein, K.; Dooley, S.; Fischerer, S.; Ochs, B.G.; Flesch, I.; et al. BMP9 a possible alternative drug for the recently withdrawn BMP7? New perspectives for (re-)implementation by personalized medicine. Arch. Toxicol. 2017, 91, 1353–1366. [CrossRef] [PubMed]

288. Devine, J.G.; Dettori, J.R.; France, J.C.; Brodt, E.; McGuire, R.A. The use of rhBMP in spine surgery: Is there a cancer risk? Evid. Based Spine Care J. 2012, 3, 35–41. [CrossRef] [PubMed]

289. Carragee, E.J.; Chu, G.; Rohatgi, R.; Hurwitz, E.L.; Weiner, B.K.; Yoon, S.T.; Comer, G.; Kopjar, B. Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis. J. Bone Joint Surg. Am. 2013, 95, 1537–1545. [CrossRef] [PubMed]

290. Epstein, N.E. Basic science and spine literature document bone morphogenetic protein increases cancer risk. Surg. Neurol. Int. 2014, 5 (Suppl. 15), S552–S560. [CrossRef] [PubMed]

291. Dettori, J.R.; Chapman, J.R.; DeVine, J.G.; McGuire, R.A.; Norvell, D.C.; Weiss, N.S. The Risk of Cancer With the Use of Recombinant Human Bone Morphogenetic Protein in Spine Fusion. Spine 2016, 41, 1317–1324. [CrossRef] [PubMed]

292. Blanquaert, F.; Saffar, J.L.; Colombier, M.L.; Carpentier, G.; Barritault, D.; Caruelle, J.P. Heparan-like molecules induce the repair of skull defects. Bone 1995, 17, 499–506. [CrossRef]

293. Uludag, H.; D’Augusta, D.; Palmer, R.; Timony, G.; Wozney, J. Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model. J. Biomed. Mater. Res. 1999, 46, 193–202. [CrossRef]

294. Lafont, J.; Blanquaert, F.; Colombier, M.L.; Barritault, D.; Carueelle, J.P. Kinetic study of early regenerative effects of RGTA11, a heparan sulfate mimetic, in rat craniotomy defects. Calcif. Tissue Int. 2004, 75, 517–525. [CrossRef] [PubMed]

295. Wang, C.; Poon, S.; Murali, S.; Koo, C.Y.; Bell, T.J.; Hinkley, S.F.; Yeong, H.; Bhakoo, K.; Nurcombe, V.; Cool, S.M. Engineering a vascular endothelial growth factor 165-binding heparan sulfate for vascular therapy. Biomaterials 2014, 35, 6776–6786. [CrossRef]

296. Khan, S.; Fung, K.W.; Rodriguez, E.; Patel, R.; Gor, J.; Mulloy, B.; Perkins, S.J. The solution structure of heparan sulfate differs from that of heparin: Implications for function. J. Biol. Chem. 2013, 288, 27737–27751. [CrossRef] [PubMed]

297. Zhang, L. Glycosaminoglycan (GAG) biosynthesis and GAG-binding proteins. Prog. Mol. Biol. Transl. Sci. 2010, 93, 1–17. [PubMed]

298. Lindahl, U.; Kjellen, L. Pathophysiology of heparan sulphate: Many diseases, few drugs. J. Intern. Med. 2013, 273, 555–571. [CrossRef] [PubMed]

299. Krusius, T.; Ruoslahti, E. Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. Proc. Natl. Acad. Sci. USA 1986, 83, 7683–7687. [CrossRef] [PubMed]

300. Ng, L.; Grodzinsky, A.J.; Patwari, P.; Sandy, J.; Plaa, A.; Ortiz, C. Individual cartilage aggrecan macromolecules and their constituent glycosaminoglycans visualized via atomic force microscopy. J. Struct. Biol. 2003, 143, 242–257. [CrossRef] [PubMed]

301. Gibson, B.G.; Briggs, M.D. The aggrecanopathies; an evolving phenotypic spectrum of human genetic skeletal diseases. Orphanet J Rare Dis. 2016, 11, 86. [CrossRef] [PubMed]

302. Roughley, P.J.; Mort, J.S. The role of aggrecan in normal and osteoarthritic cartilage. J. Exp. Orthop. 2014, 1, 8. [CrossRef] [PubMed]

303. Kiani, C.; Chen, L.; Wu, Y.J.; Yee, A.J.; Yang, B.B. Structure and function of aggrecan. Cell Res. 2002, 12, 19–32. [CrossRef] [PubMed]

304. Chang, P.S.; McLane, L.T.; Fogg, R.; Scrimgeour, J.; Temenoff, J.S.; Granqvist, A.; Curtis, J.E. Cell Surface Access Is Modulated by Tethered Bottlebrush Proteoglycans. Biophys. J. 2016, 110, 2739–2750. [CrossRef] [PubMed]

305. SundarRaj, N.; Fite, D.; Ledbetter, S.; Chakravarti, S.; Hassell, J.R. Perlecan is a component of cartilage matrix and promotes chondrocyte attachment. J. Cell Sci. 1995, 108, 2663–2672. [PubMed]

306. Gu, G.; Deutch, A.Y.; Franklin, J.; Levy, S.; Wallace, D.C.; Zhang, J. Profiling genes related to mitochondrial function in mice treated with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Biochem. Biophys. Res. Commun. 2003, 308, 197–205. [CrossRef]
307. Echtermeyer, F.; Bertrand, J.; Dreier, R.; Meinecke, I.; Neugebauer, K.; Fuerst, M.; Lee, Y.J.; Song, Y.W.; Herzog, C.; Theilmeier, G.; et al. Syndecan-4 regulates ADAMTS-5 activation and cartilage breakdown in osteoarthritis. *Nat. Med.* 2009, 15, 1072–1076. [CrossRef] [PubMed]

308. Wilusz, R.E.; Defrate, L.E.; Guilak, F. A biomechanical role for perlecan in the pericellular matrix of articular cartilage. *Matrix Biol.* 2012, 31, 320–327. [CrossRef] [PubMed]

309. Li, L.; Iy, M.; Linhardt, R.J. Proteoglycan sequence. *Mol. Biosyst.* 2012, 8, 1613–1625. [CrossRef] [PubMed]

310. Caterson, B. Feli-Muir Lecture: Chondroitin sulphate glycosaminoglycans: Fun for some and confusion for others. *Int. J. Exp. Pathol.* 2012, 93, 1–10. [CrossRef] [PubMed]

311. Yayon, A.; Klagsbrun, M.; Esko, J.D.; Leder, P.; Ornitz, D.M. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* 1991, 64, 841–848. [CrossRef]

312. Yayon, A.; Klagsbrun, M.; Esko, J.D.; Leder, P.; Ornitz, D.M. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell Biol.* 1992, 12, 240–247.

313. Schaefer, T.; Roux, M.; Stuhlsatz, H.W.; Herken, R.; Coulomb, B.; Krieg, T.; Smola, H. Glycosaminoglycans modulate cell-matrix interactions of human fibroblasts and endothelial cells in vitro. *J. Cell Sci.* 1996, 109, 479–488. [PubMed]

314. Ji, Z.S.; Pitas, R.E.; Mahley, R.W. Differential cellular accumulation/retention of apolipoprotein E mediated by cell surface heparan sulfate proteoglycans. Apolipoproteins E3 and E2 greater than e4. *J. Biol. Chem.* 1998, 273, 13452–13460. [CrossRef] [PubMed]

315. Linhardt, R.J.; Toida, T. Role of glycosaminoglycans in cellular communication. *Acc. Chem. Res.* 2004, 37, 431–438. [CrossRef] [PubMed]

316. Sadir, R.; Imberty, A.; Baleux, F.; Lortat-Jacob, H. Heparan sulfate/heparin oligosaccharides protect stromal cell-derived factor-1 (SDF-1)/CXCL12 against proteolysis induced by CD26/dipeptidyl peptidase IV. *J. Biol. Chem.* 2004, 279, 43854–43860. [CrossRef] [PubMed]

317. Vives, R.R.; Lortat-Jacob, H.; Chrboveczek, J.; Fender, P. Heparan sulfate proteoglycan mediates the selective attachment and internalization of serotype 3 human adenovirus dodecahedron. *Virolology* 2004, 321, 332–340. [CrossRef] [PubMed]

318. Capurro, M.I.; Xu, P.; Shi, W.; Li, F.; Jia, A.; Filmus, J. Glypican-3 inhibits Hedgehog signaling during development by competing with patched for Hedgehog binding. *Dev. Cell* 2008, 14, 700–711. [CrossRef] [PubMed]

319. Lewis, P.N.; Pinali, C.; Young, R.D.; Meek, K.M.; Quantock, A.J.; Knupp, C. Structural interactions between collagen and proteoglycans are elucidated by three-dimensional electron tomography of bovine cornea. *Structure* 2010, 18, 239–245. [CrossRef] [PubMed]

320. Ortmann, C.; Pickhinke, U.; Exner, S.; Ohlig, S.; Lawrence, R.; Bboot, H.; Dreier, R.; Grobe, K. Sonic hedgehog processing and release are regulated by glypicanc heparan sulfate proteoglycans. *J. Cell Sci.* 2015, 128, 4462. [CrossRef] [PubMed]

321. Shriver, Z.; Capila, I.; Venkataraman, G.; Sasisekharan, R. Heparin and heparan sulfate: Analyzing structure and microheterogeneity. *Handb. Exp. Pharmacol.* 2012, 207, 159–176.

322. Mikami, T.; Kitagawa, H. Biosynthesis and function of chondroitin sulfate. *Biochim. Biophys. Acta* 2013, 1830, 4719–4733. [CrossRef] [PubMed]

323. Poullain, F.E.; Yost, H.J. Heparan sulfate proteoglycans: A sugar code for vertebrate development? *Development* 2015, 142, 3456–3467. [CrossRef] [PubMed]

324. Langford-Smith, A.; Keenan, T.D.; Clark, S.J.; Bishop, P.N.; Day, A.J. The role of complement in age-related macular degeneration: Heparan sulphate, a ZIP code for complement factor H? *J. Innate Immun.* 2014, 6, 407–416. [CrossRef] [PubMed]

325. Lamanna, W.C.; Kalus, I.; Padva, M.; Baldwin, R.J.; Merry, C.L.; Dierks, T. The heparanome—The enigma of encoding and decoding heparan sulfate sulfation. *J. Biotechnol.* 2007, 129, 290–307. [CrossRef] [PubMed]

326. Pomin, V.H.; Mullloy, B. Current structural biology of the heparin interactome. *Curr. Opin. Struct. Biol.* 2015, 34, 17–25. [CrossRef] [PubMed]

327. Sasisekharan, R.; Venkataraman, G. Heparin and heparan sulfate: Biosynthesis, structure and function. *Curr. Opin. Chem. Biol.* 2000, 4, 626–631. [CrossRef]

328. Kreuger, J.; et al. Interactions between heparan sulfate and proteins: The concept of specificity. *J. Cell Biol.* 2006, 174, 323–327. [CrossRef] [PubMed]
329. Whitelock, J.; Melrose, J. Heparan sulfate proteoglycans in healthy and diseased systems. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 2011, 3, 739–751. [CrossRef] [PubMed]

330. Nugent, M.A.; Zaia, J.; Spencer, J.L. Heparan sulfate-protein binding specificity. *Biochemistry (Moscow)* 2013, 78, 726–735. [CrossRef] [PubMed]

331. Johnson, C.E.; Crawford, B.E.; Stavridis, M.; Ten Dam, G.; Wat, A.L.; Rushton, G.; Ward, C.M.; Wilson, V.; van Kuppevelt, T.H.; Esko, J.D.; et al. Essential alterations of heparan sulfate during the differentiation of embryonic stem cells to Sox1-enhanced green fluorescent protein-expressing neural progenitor cells. *Stem Cells* 2007, 25, 1913–1923. [CrossRef] [PubMed]

332. Baldwin, R.J.; ten Dam, G.B.; van Kuppevelt, T.H.; Lacaud, G.; Gallagher, J.T.; Kouskoff, V.; Merry, C.L. Regulation of chondrogenesis by heparan sulfate and structurally related glycosaminoglycans. *J. Biol. Chem.* 2007, 282, 6241–6252. [CrossRef] [PubMed]

333. Otsuki, S.; Hanson, S.R.; Miyaki, S.; Grogan, S.P.; Kinoshita, M.; Lotz, M.K. Expression of novel extracellular sulfatases Sulf-1 and Sulf-2 in normal and osteoarthritic articular cartilage. *Arthritis Res. Ther.* 2008, 10, R61. [CrossRef] [PubMed]

334. Suchorska, W.M.; Lach, M.S.; Richter, M.; Kaczmarczyk, J.; Trzeeciak, T. Bioimaging: An Useful Tool to Monitor Differentiation of Human Embryonic Stem Cells into Chondrocytes. *Ann. Biomed. Eng.* 2016, 44, 1845–1859. [CrossRef] [PubMed]

335. Meade, K.A.; White, K.J.; Pickford, C.E.; Holley, R.J.; Marson, A.; Tillotson, D.; van Kuppevelt, T.H.; Whittle, J.D.; Day, A.J.; Merry, C.L. Immobilization of heparan sulfate on electrospun meshes to support embryonic stem cell culture and differentiation. *J. Biol. Chem.* 2013, 288, 5530–5538. [CrossRef] [PubMed]

336. Suchorska, W.M.; Zak, B.M.; Rougier, N.; Esko, J.D.; Werb, Z. Mice deficient in Ext2 lack heparan sulfate and hematopoietic differentiation. *Stem Cells* 2008, 26, 3108–3118. [CrossRef] [PubMed]

337. Baldwin, R.J.; ten Dam, G.B.; van Kuppevelt, T.H.; Lacaud, G.; Gallagher, J.T.; Kouskoff, V.; Merry, C.L. Heparan sulfate proteoglycans modulate neural specification of mouse embryonic stem cells. *Stem Cells* 2011, 29, 629–640. [CrossRef] [PubMed]

338. Stickens, D.; Zak, B.M.; Rougier, N.; Esko, J.D.; Werb, Z. Mice deficient in Ext2 lack heparan sulfate and develop exostoses. *Development* 2005, 132, 5055–5068. [CrossRef] [PubMed]

339. Matsumoto, Y.; Matsumoto, K.; Irie, F.; Fukushi, J.; Stallcup, W.B.; Yamaguchi, Y. Conditional ablation of the Nairn, A.V.; Aoki, K.; dela Rosa, M.; Porterfield, M.; Lim, J.M.; Kulik, M.; Pierce, J.M.; Wells, L.; Dalton, S.; Tiemeyer, M.; et al. Regulation of glycan structures in murine embryonic stem cells: Combined transcript profiling of glycans-related genes and glycan structural analysis. *J. Biol. Chem.* 2012, 287, 37835–37856. [CrossRef] [PubMed]

340. San Antonio, J.D.; Winston, B.M.; Tuan, R.S. Regulation of chondrogenesis by heparan sulfate and structurally related glycosaminoglycans. *Dev. Biol.* 2011, 348, 1845–1859. [CrossRef] [PubMed]

341. Faiyaz ul Haque, M.; King, L.M.; Krakow, D.; Cantor, R.M.; Rusiniak, M.E.; Swank, R.T.; Superti-Furga, A.; Vincent, T.; Hermansson, M.; Bolton, M.; Wait, R.; Saklatvala, J. Basic FGF mediates an immediate response of hematopoietic differentiation of mouse embryonic stem cells using soluble heparin and heparan sulfate saccharides. *J. Biol. Chem.* 2011, 286, 6241–6252. [CrossRef] [PubMed]

342. Paz Soldan, S.; Abbas, H.; Ahmad, W.; Ahmad, M.; et al. Mutations in orthologous genes in human syndecan-3 modulate BMP activity during limb cartilage differentiation. *Matrix Biol.* 2006, 25, 27–39. [CrossRef] [PubMed]
347. Chen, J.; Wang, Y.; Chen, C.; Lian, C.; Zhou, T.; Gao, B.; Wu, Z.; Xu, C. Exogenous Heparan Sulfate Enhances the TGF-beta3-Induced Chondrogenesis in Human Mesenchymal Stem Cells by Activating TGF-beta/Smad Signaling. *Stem. Cells Int.* 2016, 2016, 1520136. [CrossRef] [PubMed]

348. Jha, A.K.; Yang, W.; Kirn-Safran, C.B.; Farach-Carson, M.C.; jia, X. Perlecan domain I-conjugated, hyaluronic acid-based hydrogel particles for enhanced chondrogenic differentiation via BMP-2 release. *Biomaterials* 2009, 30, 6964–6975. [CrossRef] [PubMed]

349. Sadatsuki, R.; Kaneko, H.; Kinoshita, M.; Futami, I.; Nonaka, R.; Culley, K.L.; Otero, M.; Hada, S.; Goldring, M.B.; Yamada, Y.; et al. Perlecan is required for the chondrogenic differentiation of synovial mesenchymal cells through regulation of Sox9 gene expression. *J. Orthop. Res.* 2017, 35, 837–846. [CrossRef] [PubMed]

350. Kim, S.H.; Turnbull, J.; Guimond, S. Extracellular matrix and cell signalling: The dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J. Endocrinol.* 2011, 209, 139–151. [CrossRef] [PubMed]

351. Khoshgoftar, M.; Ito, K.; van Donkelaar, C.C. The influence of cell-matrix attachment and matrix development on the micromechanical environment of the chondrocyte in tissue-engineered cartilage. *Tissue Eng. Part A* 2014, 20, 3112–3121. [CrossRef] [PubMed]

352. Hayes, A.J.; Shu, C.C.; Lord, M.S.; Little, C.B.; Whitelock, J.M.; Melrose, J. Pericellular colocalisation and interactive properties of type VI collagen and perlecan in the intervertebral disc. *Eur. Cell Mater.* 2016, 32, 40–57. [CrossRef] [PubMed]

353. Schminke, B.; Frese, J.; Bode, C.; Goldring, M.B.; Miosge, N. Laminins and Nidogens in the Pericellular Matrix of Chondrocytes: Their Role in Osteoarthritis and Chondrogenic Differentiation. *Am. J. Pathol.* 2016, 186, 410–418. [CrossRef] [PubMed]

354. Xu, X.; Li, Z.; Leng, Y.; Neu, C.P.; Calve, S. Knockdown of the pericellular matrix molecule perlecan lowers in situ cell and matrix stiffness in developing cartilage. *Dev. Biol.* 2016, 418, 242–247. [CrossRef] [PubMed]

355. Hardingham, T.; Bayliss, M. Proteoglycans of articular cartilage: Changes in aging and in joint disease. *Semin. Arthritis Rheum.* 1990, 20 (Suppl. 1), 12–33. [CrossRef]

356. Han, C.; Yan, D.; Belenkaya, T.Y.; Lin, X. Drosophila glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc. *Development* 2005, 132, 667–679. [CrossRef] [PubMed]

357. Ayerst, B.I.; Day, A.J.; Nurcombe, V.; Cool, S.M.; Merry, C.L. New strategies for cartilage regeneration exploiting selected glycosaminoglycans to enhance cell fate determination. *Biochem. Soc. Trans.* 2014, 42, 703–709. [CrossRef] [PubMed]

358. Coombe, D.R.; Kett, W.C. Heparan sulfate-protein interactions: Therapeutic potential through structure-function insights. *Cell Mol. Life Sci.* 2005, 62, 410–424. [CrossRef] [PubMed]

359. Zaia, J. Glycosaminoglycan glycomics using mass spectrometry. *Mol. Cell Proteomics* 2013, 12, 885–892. [CrossRef] [PubMed]

360. Fu, L.; Suflita, M.; Linhardt, R.J. Bioengineered heparins and heparan sulfates. *Adv. Drug Deliv. Rev.* 2016, 97, 237–249. [CrossRef] [PubMed]

361. Puvirajesinghe, T.M.; Turnbull, J.E. Glycoarray Technologies: Deciphering Interactions from Proteins to Live Cell Responses. *Microarrays (Basel)* 2016, 5. [CrossRef] [PubMed]

362. Smith, B.D.; Grande, D.A. The current state of scaffolds for musculoskeletal regenerative applications. *Nat. Rev. Rheumatol.* 2015, 11, 213–222. [CrossRef] [PubMed]

363. Kimura, T.; Yasui, N.; Ohsawa, S.; Ono, K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. *Clin. Orthop. Relat. Res.* 1984, 186, 231–239.

364. Schulz, R.M.; Zscharnack, M.; Hanisch, I.; Geiling, M.; Hepp, P.; Bader, A. Cartilage tissue engineering by collagen matrix associated bone marrow derived mesenchymal stem cells. *Biomed. Mater. Eng.* 2008, 18 (Suppl. 1), S55–S70. [PubMed]

365. Bertolo, A.; Arcolino, F.; Capossela, S.; Taddei, A.R.; Baur, M.; Potzel, T.; Stoyanov, J. Growth Factors Cross-Linked to Collagen Microcarriers Promote Expansion and Chondrogenic Differentiation of Human Mesenchymal Stem Cells. *Tissue Eng. Part A* 2015, 21, 2618–2628. [CrossRef] [PubMed]

366. Vazquez-Portalatin, N.; Kilner, C.E.; Panitch, A.; Liu, J.C. Characterization of Collagen Type I and II Blended Hydrogels for Articular Cartilage Tissue Engineering. *Biomacromolecules* 2016, 17, 3145–3152. [CrossRef] [PubMed]
367. Vazquez-Portalatin, N.; Klimer, C.E.; Panitch, A.; Liu, J.C. Knee joint preservation with combined neutralising high tibial osteotomy (HTO) and Matrix-induced Autologous Chondrocyte Implantation (MACI) in younger patients with medial knee osteoarthritis: A case series with prospective clinical and MRI follow-up over 5 years. Knee 2012, 19, 431–439.

368. Ventura, A.; Memeo, A.; Borgo, E.; Terzaghi, C.; Legnani, C.; Albisetti, W. Repair of osteochondral lesions in the knee by chondrocyte implanting using the MACI(R) technique. Knee Surg. Sports Traumatol. Arthrosc. 2012, 20, 121–126. [CrossRef] [PubMed]

369. Deponti, D.; Di Giancamillo, A.; Gervaso, F.; Domenicucci, M.; Domenechini, C.; Sannino, A.; Peretti, G.M. Collagen scaffold for cartilage tissue engineering: The benefit of fibrin glue and the proper culture time in an infant cartilage model. Tissue Eng. Part A 2014, 20, 1113–1126. [CrossRef] [PubMed]

370. Kusano, T.; Jakob, R.P.; Magnussen, R.A.; Hoogewoud, H.; Jacobi, M. Treatment of isolated chondral and osteochondral defects in the knee by autologous matrix-induced chondrogenesis (AMIC). Knee Surg. Sports Traumatol. Arthrosc. 2012, 20, 2109–2115. [CrossRef] [PubMed]

371. Gille, J.; Behrens, P.; Volpi, P.; de Girolamo, L.; Reiss, E.; Zoch, W.; Anders, S. Outcome of Autologous Matrix Induced Chondrogenesis (AMIC) in cartilage knee surgery: Data of the AMIC Registry. Arch. Orthop. Trauma Surg. 2013, 133, 87–93. [CrossRef] [PubMed]

372. Benthen, J.P.; Behrens, P. Nanostructured autologous matrix induced chondrogenesis (NAMIC(c))—Further development of collagen membrane chondrogenesis combined with subchondral drilling: A technical note. Knee 2015, 22, 411–415. [CrossRef] [PubMed]

373. Levingstone, T.J.; Ramesh, A.; Brady, R.T.; Brama, P.A.; Kearney, C.; Gleeson, J.P.; O’Brien, F.J. Cell-free multi-layered collagen-based scaffolds demonstrate layer specific regeneration of functional osteochondral tissue in caprine joints. Biomaterials 2016, 87, 69–81. [CrossRef] [PubMed]

374. Levingstone, T.J.; Thompson, E.; Matsiko, A.; Schepens, A.; Gleeson, J.P.; O’Brien, F.J. Multi-layered collagen-based scaffolds for osteochondral defect repair in rabbits. Acta Biomater. 2016, 32, 149–160. [CrossRef] [PubMed]

375. Yuan, X.; Zhou, M.; Gough, J.; Glidle, A.; Yin, H. A novel culture system for modulating single cell geometry in 3D. Acta Biomater. 2015, 24, 228–240. [CrossRef] [PubMed]

376. Nehrer, S.; Domayer, S.; Dorotka, R.; Schatz, K.; Bindreiter, U.; Kotz, R. Three-year clinical outcome after chondrocyte transplantation using a hyaluronan matrix for cartilage repair. Eur. J. Radiol. 2006, 57, 3–8. [CrossRef] [PubMed]

377. Gobbi, A.; Kon, E.; Berruto, M.; Filardo, G.; Delcogliano, M.; Boldrini, L.; Bathen, L.; Maracci, M. Patellofemoral full-thickness chondral defects treated with second-generation autologous chondrocyte implantation: Results at 5 years’ follow-up. Am. J. Sports Med. 2009, 37, 1083–1092. [CrossRef] [PubMed]

378. Brix, M.O.; Stelzeneder, D.; Chiari, C.; Koller, U.; Nehrer, S.; Dorotka, R.; Windhager, R.; Domayer, S.E. Treatment of Full-Thickness Chondral Defects With Hyalograft C in the Knee: Long-term Results. Am. J. Sports Med. 2014, 42, 1426–1432. [CrossRef] [PubMed]

379. Methot, S.; Changoo, A.; Tran-Khanh, N.; Hoemann, C.D.; Stanish, W.D.; Restrepo, A.; Shive, M.S.; Buschmann, M.D. Osteochondral Biopsy Analysis Demonstrates That BST-CarGel Treatment Improves Structural and Cellular Characteristics of Cartilage Repair Tissue Compared With Microfracture. Cartilage 2016, 7, 16–28. [CrossRef] [PubMed]

380. Merkle, H.P. Drug delivery’s quest for polymers: Where are the frontiers? Eur. J. Pharm. Biopharm. 2015, 97, 293–303. [CrossRef] [PubMed]

381. Liu, X.; Holzwarth, J.M.; Ma, P.X. Functionalized synthetic biodegradable polymer scaffolds for tissue engineering. Macromol. Biosci. 2012, 12, 911–919. [CrossRef] [PubMed]

382. Temenoff, J.S.; Mikos, A.G. Review: Tissue engineering for regeneration of articular cartilage. Biomaterials 2000, 21, 431–440. [CrossRef]

383. Gentile, P.; Chiono, V.; Carmagnola, I.; Hatton, P.V. An overview of poly(lactic-co-glycolic) acid (PLGA)-based biomaterials for bone tissue engineering. Int. J. Mol. Sci. 2014, 15, 3640–3659. [CrossRef] [PubMed]

384. Zhang, X.; Wu, Y.; Pan, Z.; Sun, H.; Wang, J.; Yu, D.; Zhu, S.; Dai, J.; Chen, Y.; Tian, N.; et al. The effects of lactate and acid on articular chondrocytes function: Implications for polymeric cartilage scaffold design. Acta Biomater. 2016, 42, 329–340. [CrossRef] [PubMed]

385. Gunatillake, P.A.; Adhikari, R. Biodegradable synthetic polymers for tissue engineering. Eur. Cell Mater. 2003, 5, 1–16, discussion 16. [CrossRef] [PubMed]
386. Farah, S.; Anderson, D.G.; Langer, R. Physical and mechanical properties of PLA, and their functions in widespread applications—A comprehensive review. *Adv. Drug Deliv. Rev.* 2016, 107, 367–392. [CrossRef] [PubMed]

387. Izal, I.; Aranda, P.; Sanz-Ramos, P.; Ripalda, P.; Mora, G.; Granero-Molto, F.; Deplaine, H.; Gomez-Ribelles, J.L.; Ferrer, G.G.; Acosta, V.; et al. Culture of human bone marrow-derived mesenchymal stem cells on of poly(L-lactic acid) scaffolds: Potential application for the tissue engineering of cartilage. *Knee Surg. Sports Traumatol. Arthrosc.* 2013, 21, 1737–1750. [CrossRef] [PubMed]

388. Moran, J.M.; Pazzano, D.; Bonassar, L.J. Characterization of polyactic acid-polyglycolic acid composites for cartilage tissue engineering. *Tissue Eng. 2003, 9,* 63–70. [CrossRef] [PubMed]

389. He, X.; Feng, B.; Huang, C.; Wang, H.; Ge, Y.; Hu, R.; Yin, M.; Xu, Z.; Wang, W.; Fu, W.; et al. Electrospun gelatin/polycaprolactone nanofibrous membranes combined with a coculture of bone marrow stromal cells and chondrocytes for cartilage engineering. *Int. J. Nanomedicine 2015, 10,* 2089–2099. [PubMed]

390. Oluwamiji, A.D.; Izadifar, Z.; Si, J.L.; Cooper, D.M.; Eames, B.F.; Chen, D.X. Modulating mechanical behaviour of 3D-printed cartilage-mimetic PCL scaffolds: Influence of molecular weight and pore geometry. *Biofabrication 2016, 8,* 025020. [CrossRef] [PubMed]

391. Deepthi, S.; Jayakumar, R. Prolonged release of TGF-beta from polyelectrolyte nanoparticle loaded macroporous chitin-polycaprolactone scaffold for chondrogenesis. *Int. J. Biol. Macromol. 2016, 93,* 1402–1409. [CrossRef] [PubMed]

392. Casper, M.E.; Fitzsimmons, J.S.; Stone, J.J.; Meza, A.O.; Huang, Y.; Ruesink, T.J.; O’Driscoll, S.W.; Reinholz, G.G. Tissue engineering of cartilage using poly-epsilon-caprolactone nanofiber scaffolds seeded in vivo with periosteal cells. *Osteoarthr. Cartil.* 2010, 18, 981–991. [CrossRef] [PubMed]

393. Pan, Z.; Ding, J. Poly(lactide-co-glycolide) porous scaffolds for tissue engineering and regenerative medicine. *Interface Focus 2012, 2,* 366–377. [CrossRef] [PubMed]

394. Haaparanta, A.M.; Jarvinen, E.; Cengiz, I.F.; Ella, V.; Kokkonen, H.T.; Kiviranta, I.; Kellomaki, M. Preparation and characterization of collagen/PLA, chitosan/PLA, and collagen/chitosan/PLA hybrid scaffolds for cartilage tissue engineering. *J. Mater. Sci. Mater. Med. 2014, 25,* 1129–1136. [CrossRef] [PubMed]

395. Chen, W.; Chen, S.; Morsi, Y.; El-Hamshary, H.; El-Newhy, M.; Fan, C.; Mo, X. Superabsorbent 3D Scaffold Based on Electrospun Nanofibers for Cartilage Tissue Engineering. *ACS Appl. Mater. Interfaces 2016, 8,* 24415–24425. [CrossRef] [PubMed]

396. Hou, J.; Fan, D.; Zhao, L.; Yu, B.; Su, J.; Wei, J.; Shin, J.W. Degradability, cytocompatibility, and osteogenesis of porous scaffolds of nanobredigite and PCL-PEG-PCL composite. *Int. J. Nanomedicine 2016, 11,* 3545–3555. [PubMed]

397. Lee, J.M.; Chae, T.; Sheikh, F.A.; Ju, H.W.; Moon, B.M.; Park, H.J.; Park, Y.R.; Park, C.H. Three dimensional poly(epsilon-caprolactone) and silk fibroin nanocomposite fibrous matrix for artificial dermis. *Mater. Sci. Eng. C Mater. Biol. Appl. 2016, 68,* 758–767. [CrossRef] [PubMed]

398. Sonomoto, K.; Yamaoka, K.; Kaneko, H.; Yamagata, K.; Sakata, K.; Zhang, X.; Kondo, M.; Zenke, Y.; Sabanai, K.; Nakayamada, S.; et al. Spontaneous Differentiation of Human Mesenchymal Stem Cells on Poly-Lactic-Co-Glycolic Acid Nano-Fiber Scaffold. *PLoS ONE 2016, 11,* e0153231. [CrossRef] [PubMed]

399. Wang, X.; Ding, B.; Li, B. Biomimetic electrospun nanofibrous structures for tissue engineering. *Mater. Today (Kidlington) 2013, 16,* 229–241. [CrossRef] [PubMed]

400. Bonzani, I.C.; George, J.H.; Stevens, M.M. Novel materials for bone and cartilage regeneration. *Curr. Opin. Chem. Biol. 2006, 10,* 568–575. [CrossRef] [PubMed]

401. Holmes, B.; Castro, N.J.; Zhang, I.G.; Zussman, E. Electrospun fibrous scaffolds for bone and cartilage tissue generation: Recent progress and future developments. *Tissue Eng. Part B 2012, 18,* 478–486. [CrossRef] [PubMed]

402. Wise, J.K.; Yarin, A.L.; Megaridis, C.M.; Cho, M. Chondrogenic differentiation of human mesenchymal stem cells on oriented nanofibrous scaffolds: Engineering the superficial zone of articular cartilage. *Tissue Eng. Part A 2009, 15,* 913–921. [CrossRef] [PubMed]

403. Kim, C.; Shores, L.; Guo, Q.; Aly, A.; Jeon, O.H.; Kim do, H.; Bernstein, N.; Bhattacharya, R.; Chae, J.J.; Yarema, K.J.; et al. Electrospun Microfiber Scaffolds with Anti-Inflammatory Tributanylated N-Acetyl-d-Glucosamine Promote Cartilage Regeneration. *Tissue Eng. Part A 2016, 22,* 689–697. [CrossRef] [PubMed]
404. Tzezana, R.; Zussman, E.; Levenberg, S. A layered ultra-porous scaffold for tissue engineering, created via a hydrospinning method. *Tissue Eng. Part C* **2008**, *14*, 281–288. [CrossRef] [PubMed]

405. Rnjak-Kovacina, J.; Weiss, A.S. Increasing the pore size of electrospun scaffolds. *Tissue Eng. Part B* **2011**, *17*, 365–372. [CrossRef] [PubMed]

406. Pham, Q.P.; Sharma, U.; Mikos, A.G. Electrospun poly(epsilon-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: Characterization of scaffolds and measurement of cellular infiltration. *Biomacromolecules* **2006**, *7*, 2796–2805. [CrossRef] [PubMed]

407. Ekaputra, A.K.; Prestwich, G.D.; Cool, S.M.; Hutmacher, D.W. Hybrid electrospun scaffolds with electrospayed hydrogels leads to three-dimensional cellularization of hybrid constructs. *Biomacromolecules* **2008**, *9*, 2097–2103. [CrossRef] [PubMed]

408. Soliman, S.; Pagliari, S.; Rinaldi, A.; Forte, G.; Fiacavento, R.; Pagliari, F.; Franzese, O.; Minieri, M.; Di Nardo, P.; Licoccia, S.; et al. Multiscale three-dimensional scaffolds for soft tissue engineering via multimodal electrospinning. *Acta Biomater.* **2010**, *6*, 1227–1237. [CrossRef] [PubMed]

409. Takanari, K.; Hong, Y.; Hashizume, R.; Huber, A.; Amoroso, N.J.; D’Amore, A.; Badylak, S.F.; Wagner, W.R. Abdominal wall reconstruction by a regionally distinct biocomposite of extracellular matrix digest and a biodegradable elastomer. *J. Tissue Eng. Regen. Med.* **2016**, *10*, 748–761. [CrossRef] [PubMed]

410. Coburn, J.; Gibson, M.; Bandalini, P.A.; Laird, C.; Mao, H.Q.; Moroni, L.; Seliktar, D.; Elisseeff, J. Biomimetics of the Extracellular Matrix: An Integrated Three-Dimensional Fiber-Hydrogel Composite for Cartilage Tissue Engineering. *Smart Struct. Syst.* **2011**, *7*, 213–222. [CrossRef] [PubMed]

411. Kim, I.L.; Mauck, R.L.; Burdick, J.A. Hydrogel design for cartilage tissue engineering: A case study with hyaluronic acid. *Biomaterials* **2011**, *32*, 8771–8782. [CrossRef] [PubMed]

412. Li, L.; He, Z.Y.; Wei, X.W.; Wei, Y.Q. Recent advances of biomaterials in biotherapy. *Regen. Biomater.* **2016**, *3*, 99–105. [CrossRef] [PubMed]

413. Ho, S.T.; Cook, S.M.; Hui, J.H.; Hutmacher, D.W. The influence of fibrin based hydrogels on the chondrogenic differentiation of human bone marrow stromal cells. *Biomaterials* **2010**, *31*, 38–47. [CrossRef] [PubMed]

414. Muller, M.; Ozturk, E.; Arlov, O.; Gatenholm, P.; Zenobi-Wong, M. Algin Sulfate-Nanocellulose Bioinks for Cartilage Bioprinting Applications. *Ann. Biomed. Eng.* **2017**, *45*, 210–223. [CrossRef] [PubMed]

415. Hamidi, M.; Azadi, A.; Rafiei, P. Hydrogel nanoparticles in drug delivery. *Adv. Drug Deliv. Rev.* **2008**, *60*, 1638–1649. [CrossRef] [PubMed]

416. Koutsopoulos, S.; Unsworth, L.D.; Nagai, Y.; Zhang, S. Controlled release of functional proteins through designer self-assembling peptide nanofiber hydrogel scaffold. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 4623–4628. [CrossRef] [PubMed]

417. Zhou, M.; Ulijn, R.V.; Gough, J.E. Extracellular matrix formation in self-assembled minimalistic bioactive hydrogels based on aromatic peptide amphiphiles. *J. Tissue Eng.* **2014**, *5*. [CrossRef] [PubMed]

418. Wang, Y.; Cheetham, A.G.; Angacian, G.; Su, H.; Xie, L.; Cui, H. Peptide-drug conjugates as effective prodrug strategies for targeted delivery. *Adv. Drug Deliv. Rev.* **2016**. [CrossRef] [PubMed]

419. Wang, Y.; Cheetham, A.G.; Angacian, G.; Su, H.; Xie, L.; Cui, H. Self-assembled peptide-based hydrogels as scaffolds for anchorage-dependent cells. *Biomaterials* **2009**, *30*, 2523–2530.

420. Zhou, M.; Ulijn, R.V.; Gough, J.E. Extracellular matrix formation in self-assembled minimalistic bioactive hydrogels based on aromatic peptide amphiphiles. *J. Tissue Eng.* **2014**, *5*. [CrossRef] [PubMed]

421. Saiani, A.; Frielinghaus, H. Self-assembly and gelation properties of a-helix versus b-sheet forming peptides. *Soft Matter* **2009**, *5*, 193–202. [CrossRef]

422. Zhou, M.; Smith, A.M.; Das, A.K.; Hodson, N.W.; Collins, R.F.; Ulijn, R.V.; Gough, J.E. Self-assembled octapeptide scaffolds for in vitro coculture. *Acta Biomater.* **2013**, *9*, 4609–4617.

423. King, P.J.; Giovanna Lizio, M.; Booth, A.; Collins, R.F.; Gough, J.E.; Miller, A.F.; Webb, S.J. A modular self-assembly approach to functionalised beta-sheet peptide hydrogel hydrogels. *Soft Matter* **2016**, *12*, 1915–1923. [CrossRef] [PubMed]

424. Coxon, T.P.; Fallows, T.W.; Gough, J.E.; Webb, S.J. A versatile approach towards multivalent saccharide displays on magnetic nanoparticles and phospholipid vesicles. *Org. Biomol. Chem.* **2015**, *13*, 10751–10761. [CrossRef] [PubMed]

425. Freudenberg, U.; Liang, Y.; Kiick, K.L.; Werner, C. Glycosaminoglycan-Based Biobuild Hydrogels: A Sweet and Smart Choice for Multifunctional Biomaterials. *Adv. Mater.* **2016**, *28*, 8861–8891. [CrossRef] [PubMed]

426. Kim, M.; Erickson, I.E.; Choudhury, M.; Pleshko, N.; Mauck, R.L. Transient exposure to TGF-beta3 improves the functional chondrogenesis of MSC-laden hyaluronic acid hydrogels. *J. Mech. Behav. Biom. Mater.* **2012**, *11*, 92–101. [CrossRef] [PubMed]
426. Getgood, A.; Henson, F.; Skelton, C.; Herrera, E.; Brooks, R.; Fortier, L.A.; Rushton, N. The Augmentation of a Collagen/Glycosaminoglycan Biphasic Osteochondral Scaffold with Platelet-Rich Plasma and Concentrated Bone Marrow Aspirate for Osteochondral Defect Repair in Sheep: A Pilot Study. Cartilage 2012, 3, 351–363. [CrossRef] [PubMed]
427. Kim, Y.J.; Kang, I.K.; Huh, M.W.; Yoon, S.C. Surface characterization and in vitro blood compatibility of poly(ethylene terephthalate) immobilized with insulin and/or heparin using plasma glow discharge. Biomaterials 2000, 21, 121–130. [CrossRef]
428. Ji, Y.; Ghosh, K.; Shu, X.Z.; Li, B.; Sokolov, J.C.; Prestwich, G.D.; Clark, R.A.; Rafailovich, M.H. Electrospun three-dimensional hyaluronic acid nanofibrous scaffolds. Biomaterials 2006, 27, 3782–3792. [CrossRef] [PubMed]
429. Wang, K.; Chen, X.; Pan, Y.; Cui, Y.; Zhou, X.; Kong, D.; Zhao, Q. Enhanced vascularization in hybrid PCL/gelatin fibrous scaffolds with sustained release of VEGF. Biomed. Res. Int. 2015, 2015, 865076. [CrossRef] [PubMed]
430. Hua, Q.; Knudson, C.B.; Knudson, W. Internalization of hyaluronan by chondrocytes occurs via receptor-mediated endocytosis. J. Cell Sci. 1993, 106, 365–375. [PubMed]
431. Sorensen, V.; Nilsen, T.; Wiedlocha, A. Functional diversity of FGF-2 isoforms by intracellular sorting. Bioessays 2006, 28, 504–514. [CrossRef] [PubMed]
432. Payne, C.K.; Jones, S.A.; Chen, C.; Zhuang, X. Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands. Traffic 2007, 8, 389–401. [CrossRef] [PubMed]
433. Mahoney, D.J.; Whittle, J.D.; Milner, C.M.; Clark, S.J.; Mulloy, B.; Buttle, D.J.; Jones, G.C.; Day, A.J.; Short, R.D. A method for the non-covalent immobilization of heparin to surfaces. Anal. Biochem. 2004, 330, 123–129. [CrossRef] [PubMed]
434. Keselowsky, B.G.; Collard, D.M.; Garcia, A.J. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. J. Biomed. Mater. Res. A 2003, 66, 247–259. [CrossRef] [PubMed]
435. Robinson, D.E.; Buttle, D.J.; Short, R.D.; McArthur, S.L.; Steele, D.A.; Whittle, J.D. Glycosaminoglycan (GAG) binding surfaces for characterizing GAG-protein interactions. Biomaterials 2012, 33, 1007–1016. [CrossRef] [PubMed]
436. Rinsch, C.L.; Chen, X.; Panchalingam, V.; Eberhart, R.C.; Wang, J.H.; Timmons, R.B. Pulsed radio frequency plasma polymerization of allyl alcohol: Controlled deposition of surface hydroxyl groups. Langmuir 1996, 12, 2995–3002. [CrossRef]
437. Marson, A.; Robinson, D.E.; Brookes, P.N.; Mulloy, B.; Wiles, M.; Clark, S.J.; Fielder, H.L.; Collinson, L.J.; Cain, S.A.; Kielty, C.M.; et al. Development of a microtiter plate-based glycosaminoglycan array for the investigation of glycosaminoglycan-protein interactions. Glycobiology 2009, 19, 1537–1546. [CrossRef] [PubMed]
438. Robinson, D.E.; Marson, A.; Short, R.D.; Buttle, D.J.; Day, A.J.; Parry, K.L.; Wiles, M.; Highfield, P.; Mistry, A.; Whittle, J.D. Surface gradient of functional heparin. Adv. Mater. 2008, 20, 1166–1169. [CrossRef] [PubMed]
439. Yang, Z.; Tu, Q.; Wang, J.; Huang, N. The role of heparin binding surfaces in the direction of endothelial and smooth muscle cell fate and re-endothelialization. Biomaterials 2012, 33, 6615–6625. [CrossRef] [PubMed]
440. Kim, M.; Hong, B.; Lee, J.; Kim, S.E.; Kang, S.S.; Kim, Y.H.; Tae, G. Composite system of PLCL scaffold and heparin-based hydrogel for regeneration of partial-thickness cartilage defects. Biomacromolecules 2012, 13, 2287–2298. [CrossRef] [PubMed]
441. Wang, J.; An, Q.; Li, D.; Wu, T.; Chen, W.; Sun, B.; El-Hamshary, H.; Al-Deyab, S.S.; Zhu, W.; Mo, X. Heparin and Vascular Endothelial Growth Factor Loaded Poly(L-lactide-co-caprolactone) Nanofiber Covered Stent-Graft for Aneurysm Treatment. J. Biomed. Nanotechnol. 2015, 11, 1947–1960. [CrossRef] [PubMed]
442. Sakler, J.P.; Liu, L. Heparin-induced osteoporosis. Br. J. Radiol. 1973, 46, 548–550. [CrossRef] [PubMed]
443. Bounameaux, H.; Schneider, P.A.; Mossaz, A.; Suter, P.; Vasey, H. Severe vasospastic reactions (ergotism) during prophylactic administration of heparin-dihydroergotamine. Vasa 1987, 16, 370–372. [PubMed]
444. Mazzotti, G.; Canalis, E.; Giustina, A. Drug-induced osteoporosis: Mechanisms and clinical implications. Am. J. Med. 2010, 123, 877–884. [CrossRef] [PubMed]
445. Bambrak, R.K.; Pham, D.C.; Zaiden, R.; Vu, H.; Tai, S. Heparin-induced thrombocytopenia. Clin. Adv. Hematol. Oncol. 2011, 9, 594–599. [PubMed]
446. Ling, L.; Camilleri, E.T.; Helledie, T.; Samsonraj, R.M.; Titmarsh, D.M.; Chua, R.J.; Dreesen, O.; Dombrowski, C.; Rider, D.A.; Galindo, M.; et al. Effect of heparin on the biological properties and molecular signature of human mesenchymal stem cells. *Gene* 2016, 576, 292–303. [CrossRef] [PubMed]

447. Ling, L.; Camilleri, E.T.; Helledie, T.; Samsonraj, R.M.; Titmarsh, D.M.; Chua, R.J.; Dreesen, O.; Dombrowski, C.; Rider, D.A.; Galindo, M.; et al. Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nat. Biotechnol.* 2008, 26, 669–675.

448. Mulloy, B.; Wu, N.; Gyapon-Quast, F.; Lin, L.; Zhang, F.; Pickering, M.C.; Linhardt, R.J.; Feizi, T.; Chai, W. Abnormally High Content of Free Glucosamine Residues Identified in a Preparation of Commercially Available Porcine Intestinal Heparan Sulfate. *Anal. Chem.* 2016, 88, 6648–6652. [CrossRef] [PubMed]

449. Liu, J.; Linhardt, R.J. Chemoenzymatic synthesis of heparan sulfate and heparin. *Nat. Prod. Rep.* 2014, 31, 1676–1685. [CrossRef] [PubMed]

450. Liu, R.; Xu, Y.; Chen, M.; Weiwer, M.; Zhou, X.; Bridges, A.S.; DeAngelis, P.L.; Zhang, Q.; Linhardt, R.J.; Liu, J. Chemoenzymatic design of heparan sulfate oligosaccharides. *J. Biol. Chem.* 2010, 285, 34240–34249. [CrossRef] [PubMed]

451. Peterson, S.; Frick, A.; Liu, J. Design of biologically active heparan sulfate and heparin using an enzyme-based approach. *Nat. Prod. Rep.* 2009, 26, 610–627. [CrossRef] [PubMed]

452. Chen, Y.; Li, Y.; Yu, H.; Sugiarto, G.; Thon, V.; Hwang, J.; Ding, L.; Hie, L.; Chen, X. Tailored design and synthesis of heparan sulfate oligosaccharide analogues using sequential one-pot multienzyme systems. *Angew. Chem. Int. Ed. Engl.* 2013, 52, 11852–11856. [CrossRef] [PubMed]

453. Xu, Y.; Cai, C.; Chandarajoti, K.; Hsieh, P.H.; Li, L.; Pham, T.Q.; Sparkenbaugh, E.M.; Sheng, J.; Key, N.S.; Pawlinski, R.; et al. Homogeneous low-molecular-weight heparins with reversible anticoagulant activity. *Nat. Chem. Biol.* 2014, 10, 248–250. [CrossRef] [PubMed]

454. Ori, A.; Wilkinson, M.C.; Fernig, D.G. A systems biology approach for the investigation of the heparin/heparan sulfate interactome. *J. Biol. Chem.* 2011, 286, 19892–19904. [CrossRef] [PubMed]

455. Kim, M.; Kim, S.E.; Kang, S.S.; Kim, Y.H.; Tae, G. The use of de-differentiated chondrocytes delivered by a heparin-based hydrogel to regenerate cartilage in partial-thickness defects. *Biomaterials* 2011, 32, 7883–7896. [CrossRef] [PubMed]

456. Kuo, Y.C.; Tsai, Y.T. Heparin-conjugated scaffolds with pore structure of inverted colloidal crystals for cartilage regeneration. *Coll. Surf. B Biointerfaces* 2011, 82, 616–623. [CrossRef] [PubMed]

457. Fernandez-Muinos, T.; Recha-Sancho, L.; Lopez-Chicon, P.; Castells-Sala, C.; Mata, A.; Semino, C.E. Bimolecular based heparin and self-assembling hydrogel for tissue engineering applications. *Acta Biomater.* 2015, 16, 35–48. [CrossRef] [PubMed]

458. Liu, Y.; Deng, L.Z.; Sun, H.P.; Xu, J.Y.; Li, Y.M.; Xie, X.; Zhang, L.M.; Deng, F.L. Sustained dual release of placental growth factor-2 and bone morphogenic protein-2 from heparin-based nanocomplexes for direct regeneration by release from heparinized mineralized collagen type I matrix scaffolds in a murine critical size bone defect model. *J. Biomed. Mater. Res. A* 2016, 104, 2126–2134. [CrossRef] [PubMed]

459. Salek-Ardakani, S.; Arrand, J.R.; Shaw, D.; Mackett, M. Heparin and heparan sulfate bind interleukin-10 and modulate its activity. *Blood* 2000, 96, 1879–1888. [PubMed]

460. Kanzaki, S.; Takahashi, T.; Kanno, T.; Ariyoshi, W.; Shinmyouzu, K.; Tujisawa, T.; Nishihara, T. Heparin inhibits BMP-2 osteogenic bioactivity by binding to both BMP-2 and BMP receptor. *J. Cell Physiol.* 2008, 216, 844–850. [CrossRef] [PubMed]

461. Hirsh, J.; Anand, S.S.; Halperin, J.L.; Fuster, V. AHA Scientific Statement: Guide to anticoagulant therapy: Heparin: A statement for healthcare professionals from the American Heart Association. *Arterioscler. Thromb. Vasc. Biol.* 2001, 21, E9. [CrossRef] [PubMed]

462. Kan, A.; Ikeda, T.; Fukai, A.; Nakagawa, T.; Nakamura, K.; Chung, U.I.; Kawaguchi, H.; Tabin, C.J. SOX11 contributes to the regulation of GDF5 in joint maintenance. *BMC Dev. Biol.* 2013, 13, 4. [CrossRef] [PubMed]
465. Fujise, M.; Takeo, S.; Kamimura, K.; Matsuo, T.; Aigaki, T.; Izumi, S.; Nakato, H. Dally regulates Dpp morphogen gradient formation in the Drosophila wing. *Development* 2003, 130, 1515–1522. [CrossRef] [PubMed]

466. Bornemann, D.J.; Duncan, J.E.; Staatz, W.; Selleck, S.; Warrior, R. Abrogation of heparan sulfate synthesis in Drosophila disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. *Development* 2004, 131, 1927–1938. [CrossRef] [PubMed]

467. Ornitz, D.M. FGFs, heparan sulfate and FGFRs: Complex interactions essential for development. *Bioessays* 2000, 22, 108–112. [CrossRef]

468. Pellegrini, L. Role of heparan sulfate in fibroblast growth factor signalling: A structural view. *Curr. Opin. Struct. Biol.* 2001, 11, 629–634. [CrossRef]

469. Kuo, W.J.; Digman, M.A.; Lander, A.D. Heparan sulfate acts as a bone morphogenetic protein coreceptor by facilitating ligand-induced receptor hetero-oligomerization. *Mol. Biol. Cell.* 2010, 21, 4028–4041. [CrossRef] [PubMed]

470. Irimura, T.; Nakajima, M.; Nicolson, G.L. Chemically modified heparins as inhibitors of heparan sulfate specific endo-beta-glucuronidase (heparanase) of metastatic melanoma cells. *Biochemistry* 1986, 25, 5322–5328. [CrossRef] [PubMed]

471. Bar-Ner, M.; Eldor, A.; Wasserman, L.; Matzner, Y.; Cohen, I.R.; Fuks, Z.; Vlodavsky, I. Inhibition of heparanase-mediated degradation of extracellular matrix heparan sulfate by non-anticoagulant heparin species. *Blood* 1987, 70, 551–557. [PubMed]

472. Parish, C.R.; Coombe, D.R.; Jakobsen, K.B.; Bennett, F.A.; Underwood, P.A. Evidence that sulphated polysaccharides inhibit tumour metastasis by blocking tumour-cell-derived heparanases. *Int. J. Cancer* 1987, 40, 511–518. [CrossRef] [PubMed]

473. Huegel, J.; Enomoto-Iwamoto, M.; Sgariglia, F.; Koyama, E.; Pacifici, M. Heparanase stimulates chondrogenesis and is up-regulated in human ectopic cartilage: A mechanism possibly involved in hereditary multiple exostoses. *Am. J. Pathol.* 2015, 185, 1676–1685. [CrossRef] [PubMed]

474. Cardin, A.D.; Weintraub, H.J. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* 1989, 9, 21–32. [CrossRef] [PubMed]

475. Ori, A.; Free, P.; Courty, J.; Wilkinson, M.C.; Fernig, D.G. Identification of heparin-binding sites in proteins by selective labeling. *Mol. Cell Proteom.* 2009, 8, 2256–2265. [CrossRef] [PubMed]

476. Chang, S.C.; Mulloy, B.; Magee, A.I.; Couchman, J.R. Two distinct sites in sonic Hedgehog combine for heparan sulfate interactions and cell signaling functions. *J. Biol. Chem.* 2011, 286, 44391–44402. [CrossRef] [PubMed]

477. Uniewicz, K.A.; Ori, A.; Ahmed, Y.A.; Yates, E.A.; Fernig, D.G. Characterisation of the interaction of neuropilin-1 with heparin and a heparan sulfate mimic library of heparin-derived sugars. *PeerJ* 2014, 2, e461. [CrossRef] [PubMed]

478. Tatsinkam, A.J.; Mulloy, B.; Rider, C.C. Mapping the heparin-binding site of the BMP antagonist gremlin by site-directed mutagenesis based on predictive modelling. *Biochem. J.* 2015, 470, 53–64. [CrossRef] [PubMed]

479. Esko, J.D.; Kimata, K.; Lindahl, U. Proteoglycans and Sulfated Glycosaminoglycans. In *Essentials of Glycobiology*; Varki, A., Ed.; Cold Spring Harbor: New York, NY, USA, 2009.

480. Powell, A.K.; Yates, E.A.; Fernig, D.G.; Turnbull, J.E. Interactions of heparin/heparan sulfate with proteins: Appraisal of structural factors and experimental approaches. *Glycobiology* 2004, 14, 17R–30R. [CrossRef] [PubMed]

481. Mosier, P.D.; Krishnasamy, C.; Kellogg, G.E.; Desai, U.R. On the specificity of heparin/heparan sulfate binding to proteins. Anion-binding sites on antithrombin and thrombin are fundamentally different. *PLoS ONE* 2012, 7, e48632. [CrossRef] [PubMed]

482. Gallagher, J. Fell-Muir Lecture: Heparan sulphate and the art of cell regulation: A polymer chain conducts the protein orchestra. *Int. J. Exp. Pathol.* 2015, 96, 203–231. [CrossRef] [PubMed]

483. Olson, S.T.; Halvorson, H.R.; Bjork, I. Quantitative characterization of the thrombin-heparin interaction. Discrimination between specific and nonspecific binding models. *J. Biol. Chem.* 1991, 266, 6342–6352. [PubMed]

484. Xu, D.; Esko, J.D. Demystifying heparan sulfate-protein interactions. *Annu. Rev. Biochem.* 2014, 83, 129–157. [CrossRef] [PubMed]
485. Petitou, M.; van Boeckel, C.A. A synthetic antithrombin III binding pentasaccharide is now a drug! What comes next? Angew. Chem. Int. Ed. Engl. 2004, 43, 3118–3133. [CrossRef] [PubMed]
486. Atha, D.H.; Lormeau, J.C.; Petitou, M.; Rosenberg, R.D.; Choay, J. Contribution of monosaccharide residues in heparin binding to antithrombin III. Biochemistry 1985, 24, 6723–6729. [CrossRef] [PubMed]
487. Xu, Y.; Wang, Z.; Liu, R.; Bridges, A.S.; Huang, X.; Liu, J. Directing the biological activities of heparan sulfate oligosaccharides using a chemoenzymatic approach. Glycobiology 2012, 22, 96–106. [CrossRef] [PubMed]
488. Lortat-Jacob, H.; Turnbull, J.E.; Grimaud, J.A. Molecular organization of the interferon gamma-binding domain in heparan sulphate. Biochem. J. 1995, 310, 497–505. [CrossRef] [PubMed]
489. Merry, C.L.; Bullock, S.L.; Swan, D.C.; Backen, A.C.; Lyon, M.; Beddington, R.S.; Wilson, V.A.; Gallagher, J.T. The molecular phenotype of heparan sulfate in the Hs2st-/- mutant mouse. J. Biol. Chem. 2001, 276, 35429–35434. [CrossRef] [PubMed]
490. Skidmore, M.A.; Guimond, S.E.; Rudd, T.R.; Fernig, D.G.; Turnbull, J.E.; Yates, E.A. The activities of heparan sulfate and its analogue heparin are dictated by biosynthesis, sequence, and conformation. Connect. Tissue Res. 2008, 49, 140–144. [CrossRef] [PubMed]
491. Catlow, K.R.; Deakin, J.A.; Wei, Z.; Delehedde, M.; Fernig, D.G.; Gherardi, E.; Gallagher, J.T.; Pavao, M.S.; Lyon, M. Interactions of hepatocyte growth factor/scatter factor with various glycosaminoglycans reveal an important interplay between the presence of iduronate and sulfate density. J. Biol. Chem. 2008, 283, 5235–5248. [CrossRef] [PubMed]
492. Raman, R.; Sasisekharan, V.; Sasisekharan, R. Structural insights into biological roles of protein-glycosaminoglycan interactions. Chem. Biol. 2005, 12, 267–277. [CrossRef] [PubMed]
493. Capila, I.; Linhardt, R.J. Heparin-protein interactions. Angew. Chem. Int. Ed. Engl. 2002, 41, 391–412. [CrossRef]
494. Thompson, L.D.; Pantoliano, M.W.; Springer, B.A. Energetic characterization of the basic fibroblast growth factor-heparin interaction: Identification of the heparin binding domain. Biochemistry 1994, 33, 3831–3840. [CrossRef] [PubMed]
495. Asensio, J.L.; Arda, A.; Canada, F.J.; Jimenez-Barbero, J. Carbohydrate-aromatic interactions. Acc. Chem. Res. 2013, 46, 946–954. [CrossRef] [PubMed]
496. Sarkar, A.; Desai, U.R. A Simple Method for Discovering Druggable, Specific Glycosaminoglycan-Protein Systems. Elucidation of Key Principles from Heparin/Heparan Sulfate-Binding Proteins. PLoS ONE 2015, 10, e0141127. [CrossRef] [PubMed]

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