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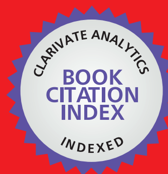

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Cartilage Tissue Engineering: the Application of Nanomaterials and Stem Cell Technology

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1. Introduction

Replacement and reconstruction of pathological or absent cartilage within the human body has been a clinical challenge for many years. The avascular nature of cartilage tissue in all areas of the human body means it has little capacity for regeneration or repair beyond the production of functionally inferior fibrocartilage. Cartilage is injured in a number of ways; in the joint region, repetitive stress can cause irreparable damage, eventually resulting in Osteoarthritis, a debilitating disorder managed only with pain medication or joint replacement. A rise in the incidence of cancer has increased the prevalence of tracheal and nasal cancers, both frequently requiring radical resection as part of aggressive treatment regimes. Congenital disorders, such as Treacher Collins syndrome and Aperts syndrome can cause severe malformation of the ear and nose. It is evident that each of these clinical scenarios involves extensive damage to crucial skeletal cartilage and it is for these reasons that a drive for advancements in cartilage tissue engineering exists.

Tissue engineering uses principles of cell biology, engineering and medicine to generate constructs that can successfully recapitulate the function of native tissues in terms of histology, mechanics and morphology. There is a need for a suitable scaffold that can provide a 3D environment for cells to proliferate and adhere. Debate still continues over the key characteristics needed for the ideal scaffold, but they are likely to differ according to the type and location of cartilage to be engineered. Should it be biodegradable/non biodegradable, natural/synthetic, and what impact do these features have on the flexibility and strength of neocartilage constructs produced? There are many scaffolds that have been extensively investigated in cartilage tissue engineering research from natural collagen and alginate, to the synthetic Polyhydroxyacids and PEG hydrogels. Nonetheless, despite advancements in scaffold design, neocartilage constructs are still mechanically inferior to their natural counterparts, and in vivo problems of poor biointegration, and deterioration in tissue quality over time limit there translation into clinical use.

Nanomaterial science has introduced new methods for improving scaffold quality. Scaffolds can now be engineered on the nanoscale, using techniques such as electrospinning and 3D fibre deposition. Likewise, the incorporation of nanoparticles into polymeric material has

allowed the addition of nanoscale features into the matrix structure. Both of these methods produce scaffolds that more closely replicate the extra cellular matrix environment found in native cartilage. It is hoped that this will increase cellular interaction with the scaffold and improve the quality of constructs produced.

With regards to the cell population to be used for engineering these constructs, there is a continued excitement over the possible application of stem cell technology. Stem cells are highly replicative and have multi lineage differentiation capacity. The traditional source of mesenchymal stem cells (precursor of chondrocytes) is bone marrow and various adjuncts to their propagation and differentiation have been explored in detail, such as growth hormones, biomaterials and environmental factors such as shear stress and oxygen tension that are important for culture techniques and bioreactor design. The discovery of new sources of mesenchymal stem cells, such as blood, adipose tissue or the synovium opens up a plethora of possibilities for clinical application, where methods of isolation and differentiation are being optimized.

In light of the numerous advancements that have been made in the last decade, this chapter aims to give a detailed account of cartilage tissue engineering strategy, looking with particular focus at the effect of scaffolds on cell growth, the evolution of stem cell technology and the expansion of bioreactor design and application . We will also explore how an integration of this revolutionary and innovative bench work can be translated into much needed clinical application in the not too distant future.

2. Cartilage in the human body

2.1 Cartilage tissue biology

Cartilage is a flexible connective tissue found in many areas of the human body, including the joints, ribs, nose, ear, trachea and intervertebral discs (Fig 1). In these regions cartilage can act as structural support, maintain shape or absorb shock during physical exercise. Unlike most other connective tissues, cartilage is largely avascular leading to hypoxic environments that limit the rate of cellular growth and tissue regeneration (115; 116). This in turn limits the capacity of cartilage to repair itself in the event of damage. The main cellular component of cartilage are chondrocytes, highly specialized cells that lie within spaces called lacunae and secrete the extracellular matrix (ECM) of cartilage . As with most connective tissues, the ECM of cartilage consists of a meshwork of macromolecules including collagens, elastin, glycoproteins and proteoglycans, each of which is present in varying amounts, depending on the type and function of cartilage. There are several cell surface receptors that allow chondrocytes to bind these proteins including the integrins, CD44, and the proteoglycan family of receptors e.g. syndecan (144).

The three types of cartilage are hyaline, elastic and fibrocartilage. Hyaline is the most abundant type, white-blue in colour and macroscopically smooth on its surface. It is present on the articular surfaces of joints and in the nasal septum. Hyaline cartilage is covered externally by a fibrous membrane known as the perichondrium, and in the joint especially, it is diffusion from the synovial fluid that provides this tissue with nutrition. It is rich in collagen type II, which forms a meshwork that encases giant aggregates of proteoglycans (Proteins with glycosaminoglycan (GAG) side chains e.g. aggrecan, biglycan, decorin in the extracellular matrix; syndecan, CD44 and fibroglycan as cell surface receptors; serglycan in intracellular tissues) (20; 21). These GAG side chains, keratan and chondroitin sulphate are able to retain water. Cyclical pressures from joint loading are crucial for normal hyaline cartilage function, and encourage the passage of water and nutrients between cartilage and

synovial fluid. Elastic cartilage however, is more flexible, due to its rich elastin fibre content that is woven into a collagen mesh (20; 21). Elastin is an insoluble protein polymer that when cross linked with desmosine and isodesmosine make up the elastin fibres themselves. This type of cartilage is also surrounded by perichondrium and is more commonly found in the pinna, Eustachian tube, larynx and epiglottis, providing crucial structural support and flexibility. The third type of cartilage is fibrocartilage, which contains abundant thick collagen type I in addition to type II, that are interlaced into a mesh work of longitudinal and circumferential fibres (20; 21). These collagen bundles impart a great ability for this type of cartilage to withstand high tensile stresses. Fibrocartilage is usually found with the intervertebral discs, sacroiliac joints, pubic symphysis and costochondral joints.

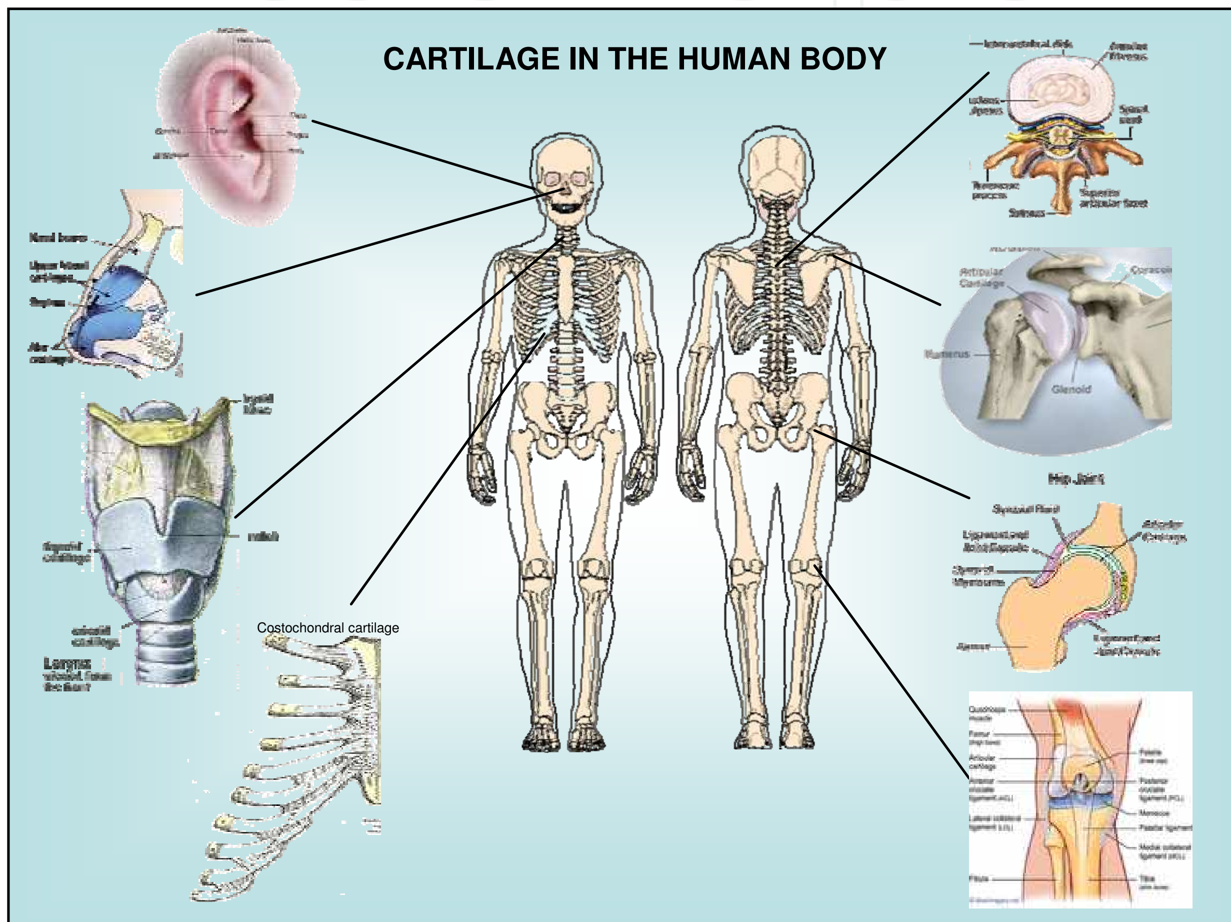

Fig. 1. A diagrammatic representation of cartilaginous regions in the human body

2.2 Development of cartilage

Central to effective tissue engineering practice is the understanding of tissue origin and development. This is based on the widely accepted hypothesis that natural tissue regeneration recapitulates developmental processes (118); hence embryological study can give an insight into the regulatory processes and patterns that govern tissue type and function, in addition to forming a foundation for understanding the degeneration and damage seen in tissues of the human body. We can only give a brief outline of the development of cartilaginous tissue specifically, however interested parties are advised to consult specific reviews (17; 70; 121) and books that devote entire chapters to this topic.

One of the epicentres of skeletal cartilage development is the growth plate which produces long bones via the cartilage template in a process known as endochondral ossification. It is important to note that this process is specific to the articular regions of bones and is followed by the eventual replacement by bony tissue. A milieu of hormones and paracrine factors govern a complex interplay of chondrocyte proliferation and differentiation, and the process can be divided into five stages, with the first three mainly being crucial for cartilage formation (144). Mesenchymal stem cells (MSCs) are first committed to becoming chondrocytes by paracrine factors that induce the expression of key transcription factors Pax 1 + scleraxis, which in turn activate cartilage specific genes. During the second stage, the committed MSCs condense into compact nodules and differentiate into chondrocytes. Chondrocytes then proliferate rapidly during the third stage, increase their cytoplasmic contents and secrete large amounts cartilage specific ECM. Their volume increases 5-10 fold, proliferation slows and they are known as hypertrophic. After this stage, the expression profile of the cells change and collagen type X and fibronectin are secreted, enabling mineralization by calcium carbonate and osteoblast infiltration to make bone. Vascular infiltration leads the way to terminal differentiation and bone development, resulting in chondrocyte apoptosis and osteoblastic differentiation. Facial cartilage development is very different, as it is embryologically derived from the cranial neural crest cells that originate from the anterior hindbrain. These cells migrate to specific locations and differentiate under the instruction of an array of Hox genes, the complexities of which are outside the scope of this chapter.

2.3 Clinical need for cartilage

Due to the limited self healing capacity of human cartilage, the repair of defects caused by degenerative joint diseases, cancer or trauma gives rise to a challenging clinical problem. In the joint region in particular, lesions of the articular cartilage are frequently associated with debilitating pain and reduced functionality. If not successfully treated long term disability can only be averted by total replacement of the joint. Damage to facial cartilaginous structures such as the nose or ear are only resolved with a prosthesis or autologous transplantation surgery that results in the formation of a donor site and frequently requires a number of revision surgeries. Large scale damage to the trachea has even less options for reconstruction with stents and tracheotomy tubes being the mainstay of treatment.

Cartilage regeneration has always been a key therapeutic target for treating articular cartilage damage in particular. Popular marrow stimulating techniques using micro-fracture or subchondral drilling of the bone have been developed to encourage the invasion of mesenchymal progenitor cells (MPC) into the affected site for spontaneous cartilage repair (94; 109). Unfortunately the outcome of such techniques varies greatly due to the lack of biological instructions for the MPCs to follow. This results in the formation of fibrocartilage which compared with hyaline tissue, has reduced durability and functionality (87; 140). The later invention of cell based therapies such as Autologous Chondrocyte Implantation (ACI) provided an important breakthrough treatment of articular cartilage damage and paved the way perhaps for more complex tissue engineering approaches with matrix assisted ACI introduced later on (16). ACI involves harvesting and propagating a population of autologous mature articular chondrocytes in vitro and re-introducing them into the defect site in cell suspension or in a supported matrix. They are then expected to lay down ECM to repair the site of injury (12; 102). Clinically the results of such procedures are good as they appear to provide symptomatic relief for patients. However, histologically the tissue produced is far inferior to native hyaline, being fibrotic in nature, again with limited

functionality and durability (19; 66; 120; 141). Further evaluations of such techniques has evidenced a strong correlation between the quality of tissue produced and the symptomatic relief of the patient from swelling and pain, once again highlighting the importance of tissue quality in cartilage regeneration. It can be postulated that these clinical breakthroughs buttressed the drive for advancements in cartilage tissue engineering technique.
It is also essential to note that the desired characteristics of engineered cartilage depend heavily on the site to be reconstructed. For instance, in tracheal constructs mechanical integrity, strength, flexibility and durability are all crucial for function, whereas in the facial region the aesthetic properties of the constructs may be equally as important. Taking the specific requirements of the tissue to be regenerated into consideration informs the tissue engineering strategy and the expected out comes of such undertakings.

2.4 Tissue engineering cartilage

Cartilage tissue engineering paradigm is based on the isolation of chondrocytes/ chondrocyte precursors from a tissue biopsy, expanding the cell number in culture, seeding them onto 3D scaffold, incubating for a period of time before placing the construct inside a patient. Many studies over the last decades have demonstrated that animal cells, when utilised in this way can produce tissues approaching the biomechanical and histological properties of native cartilage, even after implantation in vivo (3; 8; 24; 48; 58; 64; 77; 99; 110; 113; 152). However challenges do arise regarding the translation of such academic success into the clinical scenario. Challenges include isolating and propagating primary human cells, gaining relevant and reproducible construct morphology and size and ensuring good durability of the construct in vivo. Cell phenotype regulation, in vitro expansion of cell numbers, scaffold design and suitability, bioreactor design are all crucial components of the tissue engineering process that need to be optimized to advance cartilage tissue engineering from a mere academic prologue, into a clinical reality and success. These challenges will be discussed at length in the rest of this chapter.

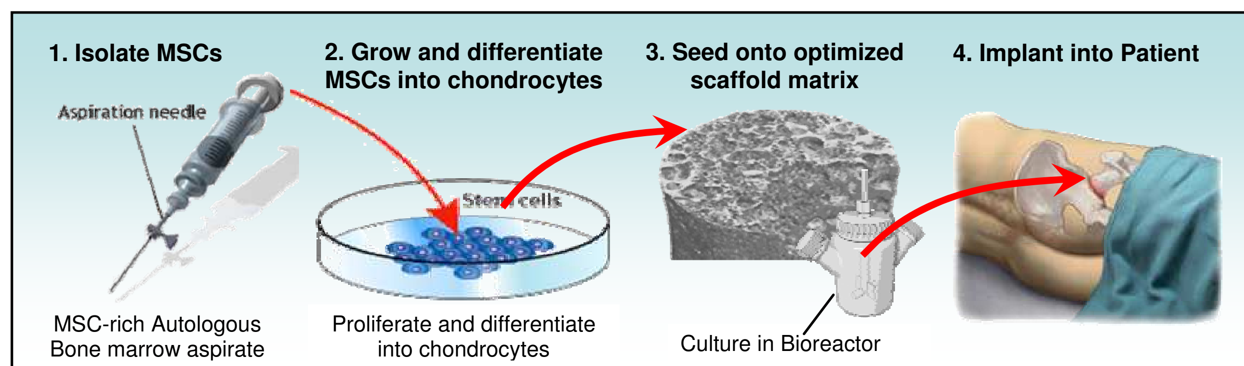

Fig. 2. A diagrammatic outline of tissue engineering strategy in cartilage tissue engineering for articular joint repair (an example of clinical usage)

3. Stem cell technology

Stem cells are unspecialised cells with a very high replication capacity. For cartilage regeneration, mesenchymal stem cells are the cell type of choice as they are multipotent stem cells, capable of differentiating into a number of lineages of the musculoskeletal

system, including osteoblasts (bone cells), chondrocytes (cartilage cells) and adipocytes (fat cells). Although not immortal, these cells are capable of expanding through many passages in culture while retaining their growth and multi-lineage potential.

MSCs can originate from various tissues including the bone marrow (11), skeletal muscle, adipose (106; 159) synovium (134), the embryo and periosteum. The optimal cell source for cartilage tissue engineering is still being identified. When selecting an ideal cell source, it is important to achieve a number of criteria, including: (i) easy access to/harvesting of the source of MSCs, (ii) extensive self-renewal or expansion capability of the cells (to generate sufficient quantities of cells for large scale tissue engineering, (iii) the ability to readily differentiate into the chondrocytic lineage when induced, and (iv) a lack of or minimal immunogenicity or 'tumourigenic' tendencies. The two most commonly used MSC sources are adipose tissue and bone marrow. Unlike other sources such as embryonic tissue, there are few ethical issues associated with harvesting and using these tissues in research and development. Additionally, bone marrow MSCs (BMSCs) and adipose derived (ADMSCs) are relatively easy to source compared with synovium-derived- or periostium-derived MSCs.

Interestingly, bone marrow is the only organ in which at least two types of stem cells exist; hematopoietic and mesenchymal stem cells (137). The MSCs are found arrayed around the central sinus in the bone marrow. The cells can be isolated from the marrow using standardised techniques and expanded in culture through many generations, while retaining their capacity to differentiate along these pathways when exposed to appropriate culture conditions. Adipose tissue is an abundant, readily available source of MSCs. The cells can be isolated from fat that has been excised or 'liposuctioned' (lipoaspirate). There are advantages and disadvantages to both techniques. Anecdotally, it is thought that excised fat provides a higher yield of MSCs compared with lipoaspirate. This is due minimal mechancal impact upon cell membranes, which would ordinarily cause cell rupture, during the isolation process. Conversely, lipoaspirate is accessible without creating a large donor site defect, a major reason for pursuing tissue engineering methods at the outset. Some studies have compared adipose-derived MSCs and bone marrow-derived MSC (107; 122) and found that both BMSCs and ADASCs are capable of chondrogenic differentiation. There is some debate over which is the superior cell source, with numerous papers highlighting each source at optimum (107).

Mesenchymal stem cells can be identified using a number of methods. These include i) examination of cell morphology, ii) FACS (fluorescence activated cell sorting) analysis to detect the expression of MSC specific markers and iii) proving their differentiation capacity by differentiating the cells into a number of lineages, namely; osteoblastic, adipocytic, and chondrocytic. For FACS analysis, the presence of MSC-specific cell surface proteins such as the following are sought; CD 105 (SH2), CD 90 (THY1) and CD 73 (SH3/4). Similarly, negative markers are used to mark and remove cells expressing cell surface proteins not typically seen on MSCs, such as CD 45, CD 34, and CD 14 (9).

3.1 Stem cell differentiation to chondrocytes

Chondrogenesis is the term used to describe the process by which a stem cell is differentiated into a mature chondrocyte and is one of the earliest morphogenetic events of embryonic development (112). The stages where introduced earlier in the section on cartilage tissue biology. They include: MSC condensation, the rise of chondroprogenitors,

chondrogenesis, terminal differentiation of progenitor cells and in skeletal development ossification (29).

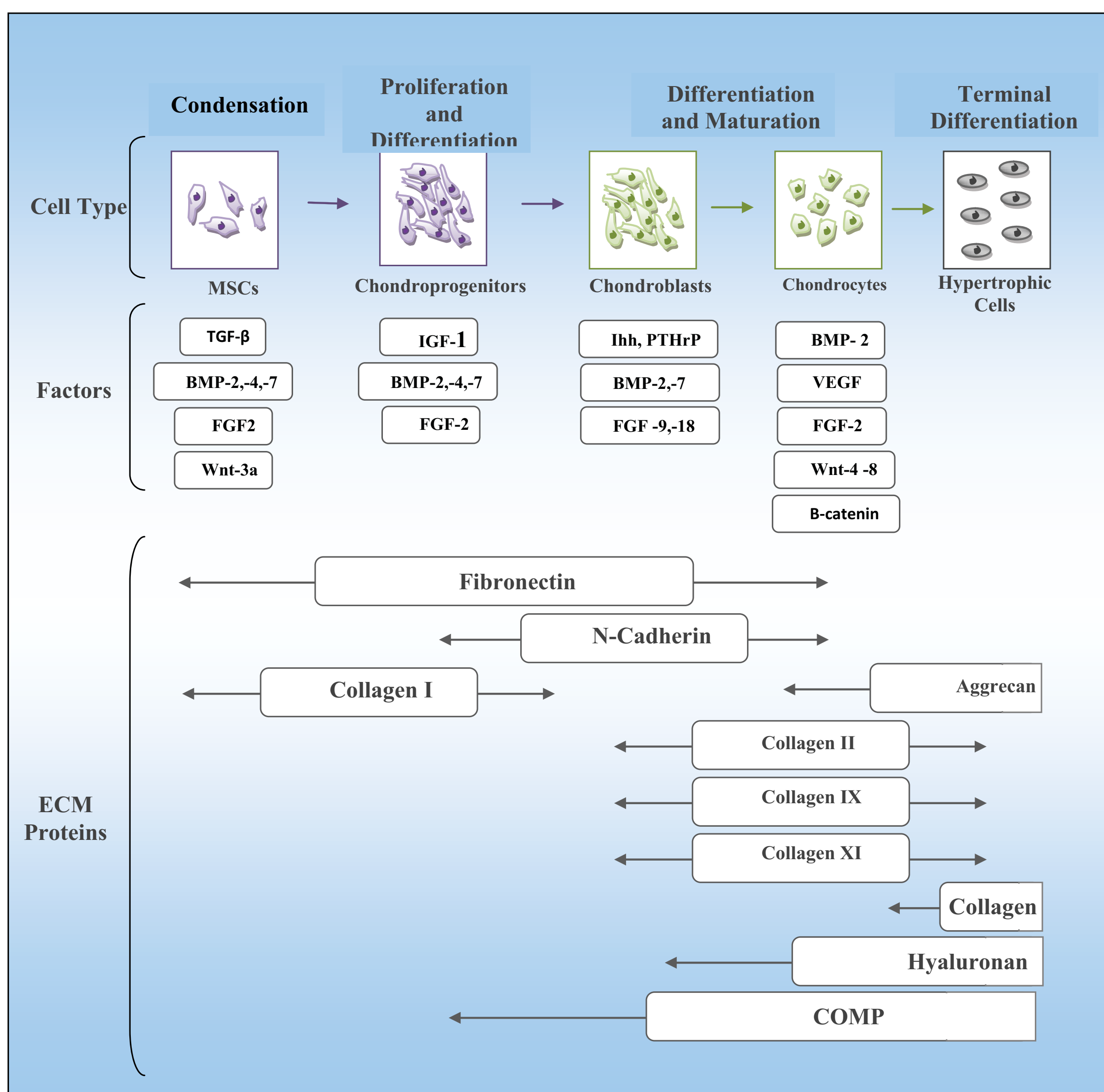

Fig. 3. Schematic diagram of the stages of chondrogenesis, the main growth factors involved in each stage and the accompanying alterations in ECM. (Adapted from Vinatier C. 2009 (144))

Condensation is directed by cell-cell and cell-matrix interactions as well as secreted factors interacting with their related receptors. Prior to condensation, the prechondrocytic MSCs secrete an extracellular matrix (ECM) which is rich in hyaluronan and collagen type I that prevents intimate cell-cell interaction (60). When condensation is initiated, there is an increase in hyaluronidase activity, thus causing a decrease in hyaluronan in the ECM. It is thought that hyaluronan facilitates cell movement, therefore, the increase in hylauronidase and subsequent decrease in hyaluronan allows for close cell-cell interactions. The establishment of the cell-cell interactions is thought to be involved in triggering one or more of the signal transduction pathways that initiate chondrogenic differentiation. Cell adhesion

molecules: N-cadherin (neural cadherin) and N-CAM (neural cell adhesion molecule) are also expressed in the condensing mesenchyme, but disappear in the differentiated cartilage, and later can be found only in the perichondrium. Perturbing the function of N-cadherin or N-CAM causes a reduction or alterations in chondrogenesis both in vitro and in vivo, which further evidences a role for these cell adhesion molecules in mediating the mesenchymal condensation step (46; 60).

As mentioned earlier, cell-matrix interactions also play an important role in mesenchymal cell condensation. Fibronectin is a component of the ECM which has the ability to regulate N-CAM. TGF-β1, one of the earliest signals in chondrogenic condensation, stimulates the synthesis of fibronectin. The expression of fibronectin is increased in areas of condensation and decreased as cytodifferentiation proceeds. Syndecan binds to fibronectin and down-regulates N-CAM thereby setting the condensation boundaries. One study showed that fibronectin mRNA undergoes alternative splicing during chondrogenesis. The isoform containing exon EIIIA is present during condensation but disappears once differentiation begins. This suggests that this isoform switching is important for cytodifferentiation to occur. A later study by the same group determined that the function of the fibronectin EIIIA exon seems to regulate mesenchymal cell spreading, therefore permitting and/or promoting adequate cell-cell interaction to take place during the condensation phase of chondrogenesis (46).

The differentiation of chondroprogenitors is characterized by the deposition of cartilage matrix containing collagens II, IX, XI and aggrecan. SOX9 is one of the earliest markers expressed in cells undergoing condensations. It is required for the expression of the type II collagen gene (Col2a1) and other cartilage-specific matrix proteins, including Col11a2 and CD-RAP prior to other matrix deposition in the cartilage anlagen. Two other Sox family members L-Sox5 and Sox6, are not expressed in early mesenchymal condensations, but are co-expressed with Sox 9 during chondrocyte differentiation. Figure 2 outlines the stages of chondrogenesis and the accompanying alterations to the ECM (46; 60).

3.2 Inducing chondrogenesis

3.2.1 Biochemical stimuli for cartilage tissue engineering

Specific biomolecules are essential for cartilage tissue engineering. The role of these biomolecules is primarily to induce chondrogenesis and to maintain the chondrocyte phenotype. There are five main families of growth factors involved in chondrogenic differentiation. These are: the transforming growth factor-β super-family (TGFβ), the fibroblast growth factor family (FGF), the insulin-like growth factor family (IGF), the wingless family (Wnt) and the hedgehog family(HH) (144). Below is a brief introduction to each growth family. However, for a more detailed description, the following references are recommended (46; 144). Figure 3 outlines the sequence in which the transcription factors are involved in each stage of chondrogenesis.

The transforming growth factor beta super-family is a family of proteins which have been shown to play a huge role in cartilage formation (11). Members include TGF-β, bone morphogenetic proteins (BMPs), inhibins and activins. All members have been shown to regulate cell growth, differentiation and apoptosis of a large number of different cell types including osteoblasts, chondrocytes, neural and epithelial cells. TGF-β is a secreted protein and exists in five isoforms TGF- β1-5. TFG- β 1,2 and 3 are thought to stimulate proteoglycans and type II collagen synthesis in chondrocytes as well as to induce

chondrogenic differentiation of MSCs (144) . Studies have also shown that TGF- β isoforms differ in their effects on various cell types. For example, TGF- β1 has been shown to be responsible for the initial cell-cell interactions between condensing progenitor cells and TGF-β2 mediates hypertrophic differentiation (32).

BMPs are also members of the TGF-β super-family and comprise of a group of 20 proteins each one playing an important role in chondrogenesis and osteogenesis during skeletal development. BMP -2,-4,-6,-7 and -13 have all proven their ability to stimulate chondrogenesis in MSCs (144) . BMP2 in particular, has been found to be expressed in the condensing mesenchyme of the developing limb (101). It regulates chondrogenic development of mesenchymal progenitors (25) as well as stimulates the synthesis of chondrocyte matrix components by articular cartilage in vitro. Even combinations of many growth factors have been to enhance chondrogenesis, for example, BMP-2 with TGF-β3 and BMP-6 with TGF-β3 have been proven to stimulate chondrogenic differentiation and result in chondrogenic lineage development (73; 127).

The FGF family is a group of growth factors consisting of 22 members. Most FGFs are secreted, except for FGF1, 2 and FGF 11 and 14 (144). Signalling by FGF18 and FGF receptor 3 have demonstrated regulation, proliferation, differentiation and matrix production of articular cartilage and growth plate chondrocytes in vivo and in vitro (45).

The IGF family is a group of proteins which have a high similarity to insulin. IGF-1 has the ability to mediate chondrogenesis by increasing proteoglycan and collagen type II production (144). Combining TGF-β3 and IGF-1, has been shown to enhance chondrogenic induction (72). One study examined the effect that IGF-1 has on the chondrogenesis of bone marrow MSCs in the presence and absence of TGF-β signalling. It showed that IGF-1 could modulate MSC chondrogenesis by stimulating proliferation, regulating cell apoptosis and inducing expression of chondrocyte markers. In addition, it proved that the chondroinductive actions of IGF-1 were equally potent to TGF-β1 and independent from the TGF-beta signalling (98). Another similar study investigated the effects of IGF-1 on TGF-β1 induced chondrogenesis. It was found that the combination of IGF-1 and TGF-β1 produced higher amounts of glycosaminoglycan than TGF-beta1 alone at 8 weeks (124).

3.2.2 Mechanotransduction in cartilage tissue engineering

In vivo, articular cartilage experiences a variety of stresses and strains on a daily bases. Thus, many groups have extensively researched into the various mechanical stimulation methods for enhancing chondrogenic differentiation and cartilage tissue engineering (67; 68; 135).Examples of the types of mechanical stimuli examined include: hydrostatic pressure (4), cyclic mechanical compression (4), shear stress (139; 143), pulsed ultrasound (130) and dynamic compressive strain (23).

It has been found that exposing differentiating MSCs to various mechanical stimuli results in a shift in the types of protein expressed during chondrogenesis. For example, the application of cyclic, mechanical compression has been shown to result in an increase in proteoglycan and collagen contents as well as a higher amount of proteoglycan-rich, extracellular matrix production. Similarly, the application of shear stress by perfusion to differentiating BM-MSCs results in an enhanced ECM deposition and an increased collagen type II production (143). Low intensity pulsed ultrasound treated cell scaffold constructs show a significant increase of chondrogenic marker gene expression and extracellular matrix deposition in differentiating human BM-MSCs (130).

Collectively, the research shows that MSCs are mechanically sensitive and the chondrogenic differentiation can be modulated and enhanced by mechanical stimulation.

4. Biomaterials

It is well established that cells reside, proliferate and differentiate inside a complex 3-dimensional (3D) ECM environment. In cartilage, chondrocytes are surrounded by a highly hydrated matrix of proteins which informs many of their phenotypic states. For example, research has shown that isolated chondrocytes will loose their differentiated phenotypes if cultured in 2-dimension (42). These chondrocytes display a shift towards a fibroblastic phenotype, evident on protein assays and histological evaluations. Type I collagen expression is increased and the typical rounded morphology of the chondrocyte becomes spindle in shape (128). This process has been shown to be reversible upon relocation to 3D matrix environments such as pellet and micro-mass culture systems, which mimic the high cell density phenomenon seen during MSC condensation, a crucial stage of cartilage development (47; 74; 92).

It is in light of this that biomaterials have been proposed as engineered 3D environments in which chondrocytes can reside. For years, material scientists along with cell biologists have worked to optimize the tissue engineering characteristics of various biomaterials. There are a number of characteristics that are thought to be necessary for general tissue engineering attempts. These include a need for biomaterials to allow adequate cell adhesion and migration, with subsequent proliferation and differentiation. The overall architecture of the scaffold should guide and frame tissue formation, whilst providing mechanical support akin to that of native tissue. The scaffold should be porous, as porosity is thought to be crucial in maintaining the phenotype of the differentiated chondrocytes, considering their preference for 3D environments. It would also allow for mass transfer of nutrients and waste products. The scaffold should also be biocompatible with the ability to integrate into surrounding native tissue.

Biomaterial scaffolds can be broadly divided into natural and synthetic scaffolds. In this section we will give a brief overview of existing natural and synthetic scaffolds used for cartilage tissue engineering research, focusing on the regulatory influence these scaffolds have on cell behaviour and the potential application of nanomaterial science to this research.

4.1 Natural

Natural materials used as bioactive scaffolds include agarose, alginate, collagen, Hyaluronic acid and acellular cartilage matrix (Table.1). The potential for clinical use of these scaffold matrices is hampered however by poor mechanical strength and flexibility, in addition to a potential for disease transfer and immune system reactivity if allogenically sourced. Their biochemical make-up leaves them prone also to host-related degradations.

Agarose: a linear polysaccharide consisting of repeating units of agarobiose, derived from Asian seaweeds and capable of supporting the chondrogenic phenotype. Its ability to form hydrogels allows it to encapsulate chondrocytes providing a 3D matrix for their growth and development. A group in Germany performed allograft transplants of chondrocytes in agarose gel into osteochondral defects in the knee of rabbits. There we no graft versus host rejections, and after 18 months, 47% of grafts had morphologically stable hyaline –like cartilage (117).

Ref.	Fabrication	Method	Cells Source	Outcome
Natural				
ALGINATE				
(49)	Chondrocytes in suspension with 2% sodium-alginate	*In vivo;* 500µl of suspension injected subcutaneously into dorsa of nude mice. Calcium chloride then injected into this area to stimulate cross linking of the scaffold. Cartilage harvested from 14 to 38 weeks	Human nasal septal chondrocytes	Gross analysis showed that 14/15 constructs resembled native human cartilage. 6 of the explants had histologically homogenous resemblance to native cartilage. The neo-constructs stained positively for Col II.
(97)	3D alginate scaffold prepared by freeze drying	*In vitro;* Cells were cultured in the alginate for 1-4 weeks in a bioreactor	Porcine articular chondrocytes	RT-PCR analysis showed the cells maintained their differentiated phenotype for up to 4 weeks. The cell also proliferated increasing from 5 x 10^5 cells to 3 x 10^7.
(151)	3D alginate gels	*In vitro;* cell/gel constructs were cultured for 0, 6, 12, 18 and 24 days	Human MSCs	Results of qRT-PCR analysis provided a temporal analysis for marker expression during chondrogenesis. Stage I (days 0–6): Col I and VI, Sox 4, and BMP-2. Stage II (days 6–12): Cartilage oligomeric matrix protein, HAPLN1, Col XI, and Sox 9. Stage III (days 12 -18): Matrilin 3, Ihh, Hbx 7, chondroadherin, and WNT 11. Stage IV (days 18–24): aggrecan, collagen IX, II, and X, osteocalcin, fibromodulin, PTHrP and alkaline phosphatise.
(91)	Alginate gel layer	*In vitro;* To evaluate the effect of low-intensity ultrasound (LIUS) on cell viability during chondrogenic differentiation	Human MSCs	When the cell/alginate construct was cultured with TGF-β1, cell viability decreased. However, addition of LIUS enhanced viability and inhibited apoptosis under the same conditions. Demonstrated by the expression profiles of apoptosis genes, p53, bax and bcl-2.
(30)	Hydrogel	*In vitro;* Chondrocytes were seeded onto alginate after 1, 2 and 3 passages in a monolayer.	Human nasal septal chondrocytes	Alginate stimulated GAG and Col I deposition supporting the chondrocytic phenotype. Results did not also support other research showing that culture with alginate beads can redifferentiate cells.

CHITOSAN				
(114)	Fibrous scaffold vs. sponge	*In vitro*; constructs analysed 3 days, 10 days and 21 days after cell seeding	Mouse BMSC line	At 10 and 21 days the cells were embedded but did not aggregate, with fibrous scaffolds containing more ECM. The cells had a round morphology. Histology revealed cell and ECM distribution was not homogenous. mRNA expression for Col II was 3 times greater for the fibrous scaffold compared with the sponge at 21 days
(22)	Chitosan scaffold and Chitosan microspheres	*In vitro;* Scaffold and microspheres used asTGF-β1 carrier to see the effect of this growth factor on chondrogenic potential	Rabbit articular chondrocytes	Encapsulation efficiency of TGF-β1 was 90.1%. TGF-β1 was released from chitosan in a multiphase fashion. TGF-β1 loaded microspheres significantly improved cell proliferation rate and Col II production, compared with controls with no microspheres or controlled TGF-β1 release.
(61)	Chitosan scaffold synthesized via freeze drying	*In vitro;* cells seeded on to chitosan of varying porosity; <10μm, 10-50μm and 70-120μm. Cultured for 28days in a rotating bioreactor	Porcine articular chondrocytes	Chitosan scaffolds remained intact compar4ed with the positive control PGA. However cartilage specific DNA levels and GAG were lower in the Chitosan groups compared with PGA. Chitosan also had the largest pores, with more Chondrocytes, but on histological analysis, the composition of cartilage produced on PGA resembled the structure of native cartilage more than chitosan constructs.
COLLAGEN				
(38)	PLGA mesh and Collagen sponge	*In vitro;* hybrid disks of PLGA/Collagen scaffold with different structures. *In vivo;* week old cultured constructs implanted into dorsa of athymic nude mice and harvested after 2, 4 and 8 weeks	Bovine articular chondrocytes	Homogenous cell distribution with natural chondrocyte morphology. Abundant ECM production. Levels of GAG and Collagen II DNA, and aggrecan mRNA increased on the scaffolds with more collagen (semi, collagen on one side of PLGA and sandwich, collagen on both sides). Semi and sandwich compared with natural articular cartilage, had a Young's modulus of 54.8% and 49.3% respectively. 76.8% and 62.7% in stiffness.

(156)	Hydrogel	*In vivo*: comparison of collagen hydrogel and collagen-alginate hydrogel. Gel injected subcutaneously into rabbit backs.	BM-MSC	Homogenous distribution of cells with chondrocyte characteristics demonstrated the chondrogenic differentiation of BM-MSCs. Both collagen hydrogel and collagen alginate hydrogel may induce chondrogenesis. Expression profile of cartilage specific genes differed between collagen hydrogel and collagen alginate, indicating that induction of chondrogenesis is materials dependent.
(153)	3D collagen sponged	*In vitro;* Cells seeded onto collagen sponges and cultured in either standard or serum free culture conditions for 1, 2 and 4 weeks	Bovine articular chondrocytes	Overall chondrogenesis in serum free culture (Nutridoma replacement) was equivalent or better than control cultures in serum. Insulin-transferrinselenium (ITS+3) serum replacement cultures were poor due to decreased cell viability. The porous 3D collagen sponges were able to maintain chondrocyte viability, shape, and synthetic activity with evidence from quantitative assays for cartilage-specific gene expression and biochemical measures of chondrogenesis.
FIBRIN				
(83)	Fibrin gel	In vivo: ACI on 30 patients using minimally invasive injection techniques. Mix of fibrin gel and chondrocytes.	Autologous adult chondrocytes	Patients evaluated 24 months post operatively using the Cincinnati knee ligament rating scores, for which 10 patients had excellent result, 17 with good results, two fair and one poor result. Further arthroscopy in 10 patients demonstrated good fill and integration in grafted areas.
(131)	PLGA/Fibrin hybrid scaffold	*In vitro;* PLGA scaffold soaked in chondrocyte-fibrin suspension (polymerized by thrombin $CaCl_2$ solution), Constructs were cultured for a maximum of 21 days.	Rabbit articular chondrocytes	Cell proliferation increased steadily until day 14, but declined by day 21. Cartilage formation evident at day 14, confirmed by the presence of cartilaginous cells embedded in basophilic ECM filled lacunae. Proteoglycan and GAG presence was confirmed. Suppression of the cart

				dedifferentiation marker Col 1 observed after 2 and 3 weeks in culture. sGAG production greater in fibrin/PLGA compared with PLGA control.
(28)	PLGA/Fibrin hybrid scaffold	*In vivo*; PLGA scaffold soaked in chondrocyte-fibrin suspension (polymerized by thrombin $CaCl_2$ solution) and constructs implanted subcutaneously into dorsum of nude mice for 4 weeks after culture for 3 weeks. Analysis performed at 1, 2 and 4 weeks.	Rabbit articular chondrocytes	Constructs maintained their shape and there was no significant difference between fibrin/PLGA and control PLGA. All exhibited smooth cartilage like properties 1, 2 and 4 weeks after implantation. Presence of proteoglycans and GAG was confirmed. The constructs were also strongly positive for Col II. Notably, sGAG production was greater on fibrin/PLGA scaffold than the control. Overall, both fibrin/PLGA and PLA showed comparable potential in sustaining the chondrogenic phenotype.
HYALURONIC ACID(HA)				
(33)	***Hyaff®-11, biodegradable polymer, nonwoven mesh	*In vitro;* Chondrocytes were harvested from OA patients and seeded onto Hyaff®. Constructs remained in culture for 28 days, analysed on day 0, 7, 14, 21 and 28.	Human Autologous chondrocytes	Viability and proliferation of OA chondrocytes similar to cells from normal subjects. Immunohistochemistry showed no signs of ageing or degeneration in cartilage produced by OA cells. The experimental groups and controls both had significantly raised Col II, Sox 9 and aggrecan. Suggests OA cells benefit from the HA rich environment.
(154)	Hydrogel (*in vitro*), beads(*in vivo*)	*In vitro* and *In vivo*; implanted into nude mice. Constructs were cultured in vitro for 2 weeks prior to implantation. Constructs remained implanted for 2 weeks.	Human MSC	Both *in vitro* and *in vivo* cultures of MSC-laden HA hydrogels enabled chondrogenesis. This was measured by the early gene expression and production of cartilage specific matrix proteins (aggrecan, Col II). HA hydrogels were compared to relatively inert poly(ethylene glycol) (PEG) hydrogels, and showed enhanced expression of cartilage specific markers

(107)	HA immobilized on surface of PLGA scaffold	*In vitro;* biodegradable macroporous PLGA scaffolds chemically conjugated to the surface exposed amine groups of the PLGA. Incubation times varied for each assay.	Bovine articular chondrocytes	Enhanced cellular attachment was observed compared with PLGA controls. GAG and total Col synthesis was significantly increased for HA/PLGA compared to the control. The HA/PLGA constructs exhibited morphological characteristics of cartilage and had cartilage specific Col II expression.
Synthetic				
PLGA- Poly(lactic-co-glycolic) acid				
(107)	PLGA scaffolds	*In vivo:* PLGA scaffolds were seeded with AD-MSC, cultured in TGFβ1 containing medium for 3 weeks, prior to implantation in the subcutaneous pockets of nude mice for 8 weeks.	Human AD-MSC	RT-PCR demonstrated the increased expression profiles of chondrospecific marker mRNA, compared with control samples after 3 weeks in vitro and 8 weeks in vivo.
(150)	HA modified porous PLGA scaffold	*In vitro;* cells seeded onto HA/PLGA scaffolds and cultured for a total of 5 days.	Human AD-MSC	The AD-MSC cultured in HA coated wells showed enhanced expression of cartilage specific mRNA. HA-modified PLGA did not affect cell adherence and viability, but did enhance gene expression after 1, 3 and 5 days in culture. GAG and Col I production enhanced after 4 weeks in culture compared with PLGA control.
(10)	PLGA scaffolds	*In vivo;* cells were pre-cultured on poly-HEMA coated dish, then seeded onto PLGA. The construct was implanted into the subcutaneous pockets of nude mice for 16 weeks.	Chondrocytes	Macroscopic signs of neo cartilage formation appeared at 8 weeks, and completed by 16 weeks. All constructs showed viable chondrocytes with normal lacunae and ECM. They stained positively for Col II. Control was a cell-free scaffold implanted into the other side of the dorsum on the same mouse.

(75)	PLGA microspheres	*In vivo;* PLGA microsphere seeded with rabbit Chondrocytes injected subcutaneously into dorsa of athymic female mice	Autologous rabbit Chondrocytes	The PLGA microsphere permitted cell adhesion. 4 and 9 weeks post-implantation there was macroscopic and histological evidence of cartilage formation on the seeded PLGA microsphere compared with nothing on the PLGA and chondrocyte controls.
(142)	PLGA porous scaffold disks	*In vivo*; MSC seeded PLGA scaffold disks implanted into 36 week old Japanese white rabbits. Constructs were harvested after 4 and 12 weeks.	Rabbit BM-MSC	Engineered cartilage from autologous BM-MSC and PLGA scaffold filled the defects in the rabbit knees. The constructs were macroscopically and histologically similar to hyaline cartilage at 12 weeks post transplantation.
PCL- Poly(carprolactone)				
(82)	3 porous PCL scaffold types investigated (1)PCL/Pluronic F127, (2)PCL collagen and (3)PCL/Pluronic F127/collagen, in addition to (4) PCL only	*In vitro;* 3 porous PCL scaffold modifications investigated (1) PCL/Pluronic F127, (2) PCL collagen and (3) PCL/Pluronic F127/collagen, in addition to (4) PCL only. Cultured for 3 weeks.	Human BM-MSC	The 3 surface treated scaffolds had higher chondrospecific DNA content than the PCL only. GAG concentrations were also higher than in the PCL only, and RT-PCR showed that Sox 9 and Col IIA1 were remarkably elevated in the modified PCLs. Notably, Col IA1 and ColI0A1 mRNA levels were lower in the three modified scaffolds than in the PCL, suggestion prevention of the dedifferentiated phenotype.
(95)	Electrospun 3D nanofibrous scaffold	*In vitro;* MSC seeded onto pre-fabricated nanofibrous scaffold for 21 days	Human BM-MSC	Histological analysis was congruent with cartilage formation when cells were grown in medium containing TGFβ1. The cartilage specific gene profile (Aggrecan, Col II and Col X) was low, but improved significantly in chondrogenic medium with TGFβ1. Col X levels were paradoxically down regulated. There was positive immunohistochemistry for cartilage specific ECM molecules.

PGA-Polyglycolic acid				
(158)	Porous PGA and high density polyethylene composite scaffold	*In vivo*; High-density polyethylene carved into cylindrical rods (internal support), with non-woven PGA sheets wrapped around the rods to form the scaffold. Implanted subcutaneously into nude mice.	Porcine BMSC	8 weeks post-implantation the constructs had formed mature cartilage with an abundant deposition of ECM on SEM. The experimental groups showed a positive histological likeness to cartilage with large number of lacunae and good expression of Col II.
(157)	PGA-HA composite scaffold	*In vivo;* MSC were seeded onto the PGA-HA and co-cultured for 72hours. There were then implanted into full thickness cartilage defects in the intercondylar fossa of rabbit femurs. Constructs were then harvested after 16 or 32 weeks of surgery.	Rabbit MSC	Grossly, the constructs demonstrated hyaline cartilage formation and at 16 weeks, there appeared to be integration with surrounding normal cartilage and subchondral bone. At 32 weeks there was no sign of degradation of the neoconstruct.
(160)	PGA vs. PLA bio-resorbable nonwoven scaffolds	*In vivo;* Cells seeded onto scaffolds and cultured for 7 days in serum free media, before implantation into subcutaneous nude mice for 6 and 12 weeks	Human articular chondrocytes	Aggrecan synthesis always higher in the PGA groups. mRNA gene expression for Col II significantly higher in the PGA groups after 6 and 12 weeks. Expression of Col X and cartilage oligomeric matrix protein increased on both scaffolds.
PEG- Poly (ethylene glycol)				
(125)	PEG-peptide copolymer gels	*In vitro;* RGD and KLER sequences chosen as motifs to modify PEG gels. (KLER is a binding site from decorin protein, known to bind strongly to Col II, RGD promotes survival of encapsulated cells). Cells were encapsulated in the PEG peptide gel and cultured for 6weeks	Human MSCs	After 14 days, cells in RGD and KLER functionalized gels produced 2.5 times as much GAG as those only containing RGD. hMSCs also produced 27x as much hydroxyproline (a major component of collagen) than scrambled sequence gel controls. Col II was more prominent in KLER gels on immunostaining and RT-PCR analysis demonstrated higher levels of Col II and aggrecan synthesis.

(111)	Hydrogel	*In vitro;* cells were encapsulated in the PEG hydrogel and allowed to free swell for 24hrs.	Bovine temporoman d-ibular chondrocytes	Condylar chondrocyte viability was maintained within the constructs during cell culture. RTPCR analysis showed the expression of cartilage specific markers, namely Col II, aggrecan and Col I was maintained

Table 1. Summary of *in vitro* and *in vivo* studies that have used various scaffolds to engineer cartilage (2005-2010). Abbreviations: AD-MSC, adipose derived mesenchymal stem cells. BMSC, bone marrow stromal cells. BM-MSC, bone marrow derived mesenchymal stem cells. Col, collagen. ECM, extra cellular matrix. GAG, glycosaminoglycan. Hbx, homeobox. Ihh, Indian hedgehog. OA, osteoarthritis. PTHrp, parathyroid hormone replacement hormone. RT-PCR, real-time polymerase chain reaction. SEM, scanning electron microscopy. sGAG, sulphated glycosaminoglycan

Alginate: derived from brown marine algae and is consists of 1, 4-linked β-D-mannuronnic and α-L-guluronic residues, which are soluble in aqueous solutions. Cross-linking with bivalent cations such as Ba^{2+} or Ca^{2+} allows it to form stable gels.
Chitin: a polysaccharide based analogue of GAG found in the exoskeleton of arthropods. Relatively unexplored bioactive scaffold for tissue engineering, perhaps because it is degraded in vivo by lysozyme; an enzyme found in many human bodily fluids.
Collagen 1 and II: As the principle ECM components of cartilage, seeded chondrocytes can bind using inherent cell-surface receptors and use standard signalling pathways to regulate proliferation and growth. Can be fabricated as a sponge, foam, or gel, but like chitin, is subject to enzymatic breakdown.
Fibrin: Can be derived from autologous blood samples, and has a comprehensive history of biocompatibility in its clinical use as a wound adhesive. Chondrocytes have integrins that can bind directly to fibrin, much like with collagen.
Gelatin: A porous substance derived from hydrolysis of collagen. Its application as a scaffold for cartilage tissue engineering is relatively unchartered.
Hyaluronic Acid: a non-sulphated GAG, found in abundantly within the cartilaginous ECM. It is crucial for maintaining the biophysical properties of the cartilage ECM for optimum chondrocyte growth and proliferation.

4.2 Synthetic

The main aim of biomimetic materials (synthetic biomaterials) is to generate 3D scaffolds that support essential cell functions in addition to mimicking the biomechanical properties of host tissues, whilst avoiding host immune responses (Table.1). These are two characteristics more difficult to find in natural scaffold alternatives. When considering clinical applications, susceptibility to vascular invasion is a key consideration and there is continued debate between groups about the need for biodegradation. Persistence and stability have been the focal aims for tissue engineering cartilage with the mechanical and biochemical properties of synthetic materials being more amenable to modification than natural scaffolds.
Polyhydroxyacids: polyhydroxyacids such as PLLA [poly (L-lactic acid)], PCL [poly (L-lactide-ε-caprolactone)] and PGA [poly (glycolic acid)] have been well studied as potential

cartilage scaffold matrices, where they are easily extruded into fibrous or open lattice sponges. PGA is reportedly highly biodegradable (5 weeks); PLLA can stay *in vivo* up to 3 years. PCL and PGA used to fabricate ear templates for tissue engineering auricular cartilage (133).

Elastomeric polyurethanes: Well documented history of use in a variety of biomedical instruments, ranging from urinary and vascular catheters to intra-aortic balloons and mammary implants. Can be fabricated in a biodegradable form, and have been shown to support chondrocyte attachment and growth.

PEG [poly (ethylene glycol)]: FDA (Food and Drugs Administration) approved, and extensive research into its ability to promote chondrogenesis

4.3 Regulatory influence of scaffolds on cell behaviour

It is widely appreciated that soluble biochemicals such as cytokines, growth factors and chemokines affect the growth and development of all tissues including cartilage. Transforming growth factor beta (TGFβ) and bone morphogenic proteins (BMPs) have been evidenced as highly potent stimulators of cartilage tissue generation (78; 96; 118). In addition to such signalling mechanisms, ECM proteins such as collagens, glycosaminoglycans and proteoglycans exert an array of instructions on cells via transmembrane receptors that affect expression and therefore, cell behaviour. Much of this instruction will crosstalk with growth factor signalling (37; 44). Additional studies have also shown chondrocytes to be particularly receptive to mechanical loading, with this parameter having been evidenced as a crucial factor in the chondrogenic differentiation of MSCs during critical cartilage development. Repetition of these loads and varying the duration and force of the load has positive effects on the structural organization of cartilage ECM (7; 62). The effects of mechanobiology on chondrogenesis have been discussed in detail in the section on stem cells.

In recent times, tissue engineering research had broadened its horizons to understand the effect of scaffold physical properties on cell behaviour. Properties considered include; roughness (88; 89; 147), micro and nanotopography (reviewed in (132)), porosity (155) and surface energy (80; 147). The stiffness of the substrate (scaffold matrix) has been demonstrated as a crucial regulator of stem cell behaviour (15; 48; 52; 119). It is thought that the stiffness or elasticity of a matrix can act as a 'passive' cue for cell processes via a phenomenon known as mechano-transduction. This is a method by which cells convert mechanical stimuli into a chemical response, thus affecting their own behaviour. For detailed reviews see (2; 54). Cells bind to the matrix using integrins. The intra cellular domain connects to the actin and myosin (contractile) cytoskeleton of the cell, and the extracellular domain to the biomaterial. When cells are bound, they apply mechanical forces onto the matrix using their contractile cytoskeleton. Integrins cluster which in turn recruits structural and signalling proteins at the site of contact between cell and matrix, known as a focal adhesion. If a matrix is relatively hard, there is more resistance to the forces applied by the cellular cytoskeleton. This results in a more organized cytoskeleton, more integrin clustering and thus focal adhesions that are greater in maturity. Comparatively, if cells are seeded onto a soft matrix, there is little resistance to counterbalance the cell forces, therefore reduced development of the actin-myosin cytoskeleton. This phenomenon is fundamental considering that changes in cytoskeletal organization affect signalling, thereby translating mechanical processes into chemical responses.

So how can this trend be used in cartilage tissue engineering technology? Let us consider the application of stem cells in tissue engineering cartilage. Stem cells extracted from human or animal sources are frequently expanded in culture. Culturing stem cells on traditional tissue culture plastic could result in preconditioning of the cells in accordance with the stiffness of the plate (51; 52). Depending on the experimental aims, it may be wiser to culture and expand on softer substrates with stiffness comparable to that of native tissue. However conflicting data has shown that stiffer substrates increase the rate of proliferation, whereas soft substrates promote the dedifferentiation of cells (15).This suggests the stiffness of the material used for cartilage tissue engineering is an important parameter not just in terms of mechanical support but also in terms of propagating chondrocyte growth and matrix deposition.

4.4 Nanomaterials

Cell coverage over a matrix layer is directly correlated to the spread of microscale ECM proteins over its surface, irrespective of the geometric patterning of such proteins (93). This theory applies at the microscale level of tissue engineering, but at the nanoscale, there is increasing evidence to indicate that cells are able to alter their behaviour differentially in response to changes in nanotopographical surfaces. These changes can be cytoskeletal or a change in morphology, focal adhesions, motility, gene expression and differentiation. Much like the mechano-transduction discussed earlier, there is support for some sort of topography-dependent transduction that communicates independent of chemical signalling from ECM molecules (35). Studies have since demonstrated that this cellular response is heavily related to the pattern and spacing of adhesive ligands (36;41;79;148).

In light of the revelation that nanotopography plays a major role in the governance of cell-matrix interactions, many physical and chemical methods have been developed to engineer geometrically defined nanopatterns on biocompatible scaffolds. Crude methods of acid treatment (85), bonding with calcium cations (63), and coating with nanoparticles (reviewed in (126)) allowed scientists to introduce nanofeatures into the surface topography of scaffolds. Surface modifications with Lanthanum phosphate ($LaPO_4$) nanoparticles increased osteoblast adhesion to traditional bioceramics; Hydroxyapatite and Tricalcium phosphate (53). Likewise with chondrocytes, the levels of adhesion increased on 70%/30% (wt) PLGA/titanium composite scaffolds manufactured to have a nanosurface (76). However the advancement of nanoscience allows more precise pattering of various nanofeatures to further affect cell behaviour. Nanofeatures now come in many forms ranging from nanopits and grooves, to nanopillars, nanodots and traditional nanoparticles. The pattern in which they are arranged is also on the nanoscale. The latest techniques used for nanosurface pattering (reviewed in (132)) include photolithography, electron beam lithography (40), Dip-pen lithography (71) and imprint lithography.

Nanopatterning to mimic the surface density and arrangement of integrin-binding epitopes as seen in the ECM has been a challenge not yet beaten. Studies have shown that integrin mediated signalling operates with a minimum surface density however; the exact spatial organization of these ligands in vivo has not been elucidated. The nearest estimate has come from a group that developed a block-copolymer micelle nanolithography technique to label surfaces with hexagonal arrays of gold nanodots coated with one RGD peptide (found in adhesive glycoproteins such as fibronectin and vitronectin) (26;27). Upon cell seeding they

found that only 28nm and 58nm spacing between the nanodots would allow adequate clustering of integrins, which are approximately 8-12nm in size (138). Additional studies on RGD-coated gold nanoparticles have shown that the velocity of migrating cells decreases with an increased particle density, with a peak velocity at circa 120nm, suggesting the boost in particle density increased levels of adhesion (6;65). Interestingly enough, research has also shown the MSC osteoblastic differentiation can be hampered by regularly arranged hexagonal nanopits arrays compared with arrays with a slight irregularity (39). Similar results were found by Biggs et al 2007, where highly ordered nanopits resulted in decreased formation and length of focal adhesions, compared with controlled disorder increasing focal adhesion formation and size (13).
With more research being conducted into the in vitro effects of surface nanopatterning on cell behaviour, there are implications for cartilage tissue engineering research. Data shows that nanostructured PLGA can accelerate chondrocyte attachment, growth and proliferation in addition to improving ECM production (76). In our lab, chondrocytes seeded onto nanocomposite polymer POSS-PCU (UCL nanoBio™) have a faster rate of proliferation compared with controls lacking the nano modification (unpublished data). And though the current research into nanomaterials and cartilage tissue engineering is just evolving, there are many lessons to be learnt from bone (80;81), skin (31) and vascular (100;108) tissue engineering research.

5. Bioreactors

Bioreactors are devices in which biological and/or biochemical processes develop under controlled and monitored environmental and operating conditions (104). It is the exceptional control over environmental conditions that makes bioreactor use particularly pertinent in tissue engineering research where specific factors need to be controlled in order to optimise tissue growth. Bioreactors can maintain physiological boundaries at desired levels, enhance nutrient and waste transport rates, and provide specific stimuli to promote optimum growth.
The use of bioreactors has provided a promising method for tackling some causes for poor research outcomes in tissue engineering practice. Restricted, unspecific, or impermanent cell differentiation and poor tissue formation/ remodelling in cartilage tissue engineering largely results from a lack of correct physical stimulation in vitro (86). For example, mechanotransduction, the transduction of mechanical stresses into biochemical signals, affects chondrocyte function. Modifying the mechanical stressors applied to cells in vitro may therefore improve the quality of tissue constructs produced. In early parts of this chapter, the effect of cyclical loading, especially within the articular region has been shown to improve the ECM content of constructs, and therefore the overall construct viability. Mimicking some of these forces in bioreactor systems could also dramatically improve tissue growth. Studies which evidenced the effect of adaptive physical stimulation on mechanotransduction, led to the development of bioreactor devices that transmit forces including shear stress, hydrostatic pressure and compression to articular cartilage in vitro (129).

5.1 Mechanical forces

Key to tissue engineering in the joint region specifically is the use of exogenous mechanical forces to simulate loading forces (exerted during daily movement and exercise), which in

turn increases the metabolic activity of and ECM production by chondrocytes. Shear stress, compressive forces, tensile forces and hydrostatic pressures are all parameters that can be modulated to influence the quality of cartilaginous constructs engineered. The effect of these mechanical forces on chondrogenesis, have been described earlier in the chapter. We will examine briefly the bioreactors that have been used to study the effects of shear stress however, the different types of bioreactors available for exerting other forces are expertly reviewed in Schulz RM 2007.

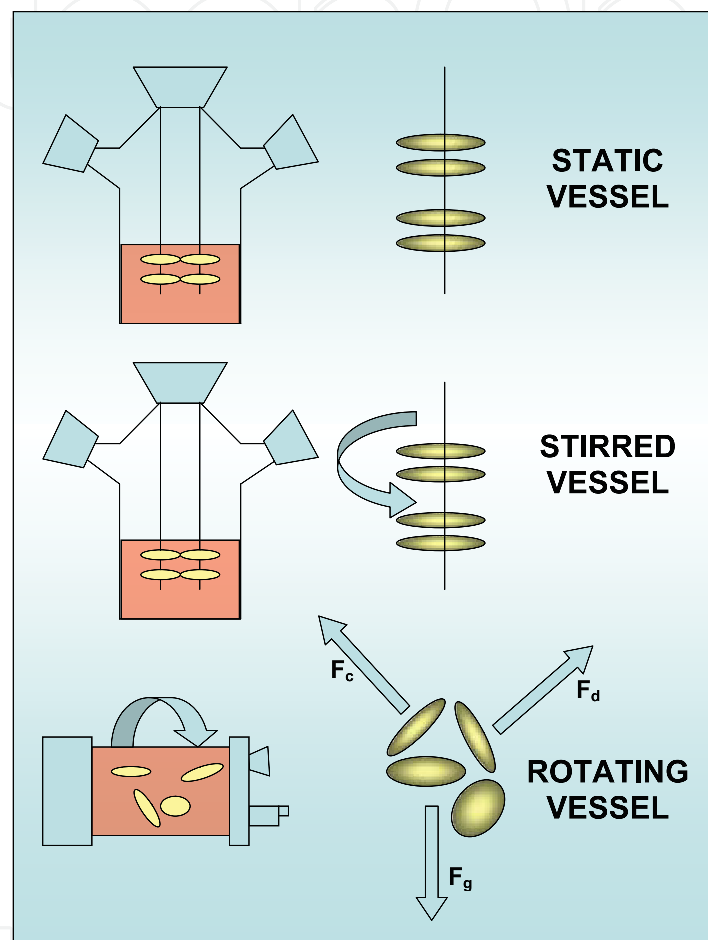

Fig. 4. Experimental appraoches to bioreactor tissue engineering. Representation of static, stirred and rotating vessels. Fc, Fd, Fg refer to centrifugal, drag and net forces respectively. (Adapted from Vunjak-Novakovic G 1999 (145))

The easiest method for examining the effects of shear stress in bioreactor systems is by placing constructs in culture either on a petri dish or in a dynamic or orbital shaker (56;57). Other methods developed have included using spinner flasks or vessels with magnetic stirrers (14;18;136). Extensive study in the nineties looked at the application of shear stress by comparing static and orbital shakers, stirred vessels with rotating vessels (Fig.4) (145). Rotating vessels are more advanced systems where constructs float freely within culture medium, whilst the whole vessel rotates around a central axis at a constants speed. Chondrocytes were seeded onto 97% porous scaffold discs and cultured in the aforementioned vessels for 8 weeks. Results showed that freely cultured constructs were larger than those cultured in static or stirred vessels. They formed cartilaginous ECM with

the greatest concentration of GAGs and collagen. Their mechanical properties where also shown to be superior.

5.2 Oxygen tension

Optimizing O_2 tension within culture systems is an area of great importance in bioreactor design. O_2 is the partial pressure of oxygen dissolved in a liquid such as blood. Cells in culture require nutrients and oxygen to proliferate and this is usually achieved through mass transport (net movement of mass from one location to another). When oxygen and nutrients are limiting factors, larger grafts tend to contain a hypoxic, necrotic centre, surrounded by a rim of viable cells (Martin I 2004). In a tissue graft, the density of cells may be higher than the distance oxygen can freely diffuse across by mass transport to provide sufficient oxygen for the inner cells; therefore they are starved of oxygen. Limited O_2 diffusion can also affect the spatial distribution of cells and as the O_2 concentration gradient decreases from the surface of the tissue compartment to its centre (34). In humans, this problem is solved by the circulatory system and thus nutrients are provided to all cells via a complex network of vessels, slowly decreasing in size the deeper into tissues they enter. It is the proximity of capillaries to somatic (body) cells that allows their mass transfer requirements to be met (105). So how is this problem solved in tissue engineering practice? The introduction of simple stirred flask bioreactors enables the mixing of oxygen and nutrients throughout the medium. So not only does it provide a shear stress which is known to be beneficial for chondrocyte growth and proliferation, but it also reduces the concentration boundary layer of oxygen at the construct surface (14;18;104). In a static culture environment, oxygen would diffuse into cells and carbon dioxide out. The medium in closest proximity to the cells would have a steadily decreasing O_2 tension with a conversely increasing CO_2 tension. This in turn limits the overall rate diffusion as O_2 moves from areas of high tension to areas of low tension. Thus if the culture medium is not circulated or replenished the rate of diffusion will decrease and eventually cease at the point where there is no longer a concentration gradient, leading to cell death.
Studies have also used bioreactors to investigate the effect of different partial pressures of O_2 and pH levels on gene and protein expression, as well as the metabolic activity of chondrocytes. Results showed chondrocyte sensitivity to acidic conditions where reduced expression of Coll Type 1, SOX9 and VEGF (vascular endothelial growth factor) were observed. Conversely in hypoxic conditions, VEGF levels were found to be higher, with a pH dependent reduction in Coll Type 1 (43). Culture in bioreactors at low oxygen tension increases the production and retention of glycosaminoglycan (GAG) within the cartilage matrix without affecting chondrocyte proliferation or collagen deposition which typically would requires higher partial pressures of O_2 (123). These studies highlight the twofold applications of bioreactors, in maximizing cell growth and tissue generation for clinical use and in research and development to investigate the effect of different biological factors on cell growth.

5.3 Growth factors

It has also bee suggested that bioreactors provide suitable environments to add growth stimulating factors to constructs to improve chondrogenesis. For example, transducing human MSCs with an adenoviral vector containing SOX9 and subjecting the construct to mechanical stimulation could increase GAG synthesis (90). Growth factor application of

BMP-2, IGF-1 and TGF-β1 in a bioreactor system can increase the compressive and tensile biomechanical properties of engineered tissue (50). The efficiency of chondrocyte proliferation from low initial seeding densities can also be enhanced by adding various growth factor combinations in to automated bioreactors systems (55). Chitosan scaffolds were used to engineer articular cartilage with the aid of a chondrogenic differentiation factor, BMP-6. Results showed that proliferated cells contained a higher value of GAG, Coll type II and DNA indicating improved chondrogenesis (1). Alternatively, inhibiting the expression of some factors, namely interleukin 6, has also been investigated with the aim of improving tissue growth in bioreactors. In 2010, Wang P et al demonstrated how high levels of interleukin – 6 have been found in osteoarthritic cartilage and suggested that inhibiting this expression may improve cartilage construct culturing in bioreactors (146).

6. Challenges for the clinical application of regenerated cartilage

Over the past two decades the amount of data on cartilage tissue engineering strategies has risen exponentially. There is now a plethora of exciting in vitro data evaluating chondrocyte/MSC seeded biomaterial constructs. Perhaps one of the most iconic studies in cartilage tissue engineering research was produced by Cao and Vacanti's group in 1996, when they implanted an auricular shaped cartilaginous construct onto the back of a mouse (24). Even with all the advancements in stem cell and biomaterial technology, the invention of various bioreactor systems, little has progressed beyond this scientifically historic event. Most constructs fail to develop beyond immature, inflexible neocartilage that lacks the durability essential to most clinical applications.

There are a number of reasons for the stagnation in translation to clinical practice. Many of which have been discussed throughout the course of this chapter. On a cellular level, reasons for poor research outcomes could also include; (i)Regenerative cells being lost through leakage of the cell suspension (149), (ii) inflammatory cytokine, matrix metalloproteinase, nitric oxide mediated apoptosis and necrosis at the site of injury. These biochemical factors are released as part of the normal inflammatory and wound healing process, especially at the interface between host and repair tissue, which can also adversely affect biointegration of the neo tissue. The use of anti-apoptotic factors would be crucial in maintaining cell numbers but also in creating a favourable environment for biointegration (5). The poor migration capacity of chondrocytes could also be responsible for hampered infiltration of repair tissue into the host environment. The naturally slow rates of chondrocyte ECM production could slow down integration as well disparities in the organization of neocartilage matrix compared with the zonal arrangement of native cartilage tissue (69;84). Dedifferentiation of chondrogenic cells is another problem, and is likely responsible to the highly fibrotic nature of neocartilage produced, suggesting that over time, cells may have dedifferentiated into fibroblasts or incompletely differentiated into chondrocytes. Solutions would include seeding with cells that have been fully differentiated in vitro, but again there would be difficulties with motility, proliferation and shelf life.

In addition to the cell based scientific problems associated with cartilage engineering tissue research, ambiguous regulatory guidelines currently hamper the flow of development from laboratories to clinics and operating theatres. The EU regulation on Advanced Therapy Medicinal Products (ATMP), which includes tissue engineered constructs, is still in its infancy having only been formally established in December 2008. ATMP regulation aims to proved a coherent and tailored framework for tissue engineered products, however the

nascent and fast growing nature of the tissue engineering field means that there is a constant threat of irrelevance over the guidelines developed under this regulation. Tissue engineering technology needs to reach a level of quality controlled and quality assured reproducibility to allow for not just clinical efficiency, but also commercial viability. Methods of stem cell differentiation, cell seeding, scaffold fabrication and bioreactor development/implementation all need to be governed by Good Manufacturing Practice (GMP). Additionally, methods of commercialization ought to be better established, to avoid uncertainty in the markets, improve regulatory approval and clinical uptake/use (103).

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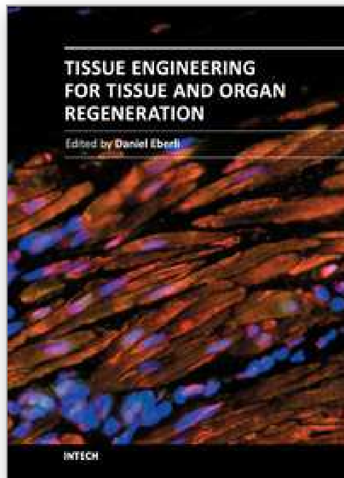

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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