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Review

Chondrogenesis of mesenchymal stem cells for cartilage tissue engineering

Oliver F.W. Gardner[1,2], Charles W. Archer[2], Mauro Alini[1] and Martin J. Stoddart[1]
[1]Musculoskeletal Regeneration, AO Research Institute, Davos Platz, Switzerland and
[2]Cardiff School of Biosciences, Museum Avenue, Cardiff, UK

Summary. Despite its remarkable ability to resist mechanical loading, articular cartilage is not capable of mounting a useful reparative reaction in response to damage caused by trauma or disease. As a result numerous surgical and medical approaches have been developed to aid the healing of articular cartilage. Despite the success of surgical techniques such as microfracture, recently attentions have been turned to cell based therapies such as autologous chondrocyte implantation (ACI). ACI has produced encouraging results, however better results may be achievable through an evolution of this surgical approach. Since the first generation of ACI techniques changes have been made in the technique e.g. the introduction of collagen membranes instead of periosteal flaps, and more recently the use of collagen scaffolds for cellular delivery. The procedure has also moved on from being performed as an open operation and can now be performed arthroscopically. Despite these advances the procedure still uses chondrocytes harvested from the joint being repaired. These cells are vulnerable to dedifferentiation during the required *in vitro* expansion, and as a result may not be capable of producing repair tissue once implanted back into the joint. Mesenchymal stem cells (MSCs) may provide a dedifferentiation resistant alternative to chondrocytes. MSCs would also allow for the use of one arthroscopic operation on the affected joint, as opposed to the two operations that are currently required for ACI.

Key words: Cartilage repair, Mesenchymal stem cells, Tissue engineering, Chondrogenesis

Offprint requests to: Martin Stoddart, Musculoskeletal Reveneration, AO Research Institute, Davos Platz, Switzerland. e-mail: martin.stoddart@aofoundation.org

Introduction

As the bearing material of diarthrodial joints articular cartilage can cope with loads that in the hip joint can reach 18 MPa. However, despite this capacity to dissipate and absorb load, cartilage is very susceptible to damage through trauma or disease, once damaged it is all but incapable of effecting a repair (Hodge et al., 1986). The susceptibility of cartilage to damage and its poor reparative response means that even small lesions can produce significant amounts of pain, joint stiffness, immobility and, over time, increases the risk of osteoarthritis (Fischer et al., 2010; Madry et al., 2010). The lack of regeneration demonstrated by articular cartilage was first realised more than two hundred and fifty years ago by the anatomist and surgeon William Hunter (Hunter, 1743). For a long time, it has been the goal of surgeons to develop a reliable method to repair damaged articular cartilage. These techniques have ranged from debris removal techniques, such as debridement and lavage developed in the 1940's, to osteochondral transplant techniques, marrow stimulation techniques and the latest generation of cell based tissue engineering techniques such as autologous chondrocyte implantation (Insall, 1967; Brittberg et al., 1994; Redman et al., 2005).

Chondrocytes account for a mere 5% of the total volume of articular cartilage, the rest is a dense extracellular matrix consisting mainly of two macromolecules, type II collagen and the aggregating proteoglycan aggrecan. These two molecules account for 90% of the dry weight of cartilage (Kisiday et al., 2009; Freyria and Mallein-Gerin, 2012). The other substance found in large amounts in cartilage is water; this makes up 70-80% of cartilages wet weight and is drawn in to the tissue by the large amounts of negative charge found on the aggrecan molecule's glycosaminoglycan chains

(Elder and Athanasiou, 2009). It is the presence of large amounts of water within cartilage combined with the organisation of the aggrecan and type II collagen that allow it to resists the large loads that it is exposed to (Becerra et al., 2010).

Articular cartilage is constructed of four layers; the surface zone, middle zone, deep zone and calcified zone (Freyria and Mallein-Gerin, 2012). Each zone has a different macromolecular arrangement, and the chondrocyte morphology and organisation is specific to each zone (Freyria and Mallein-Gerin, 2012). The surface zone contains flattened chondrocytes that express a natural lubricating molecule called lubricin or proteoglycan 4 (PRG4), which helps produce articular cartilage's almost frictionless surface (Klein et al., 2009). In the middle zone, chondrocytes become more rounded, and are spread unevenly throughout the matrix (Klein et al., 2009). The chondrocytes of the deep zone are spherical and organised into clear columns. The calcified layer acts as a transition layer between the articular cartilage and the bone beneath it. The chondrocytes of the calcified layer contain little cytoplasm or cellular machinery (James and Uhl, 2001). The type II collagen fibres within articular cartilage form arch like structures referred to as the arcades of Benninnghoff, the fibres are perpendicular to the surface in the deep zone, and parallel to the surface in the superficial zone (Klein et al., 2009). In the mid-zone the collagen forms a dense mesh as overlapping fibres curve to change from being vertical in the deep zone to horizontal in the superficial zone (Becerra et al., 2010). The horizontal fibres in the superficial zone give the surface of cartilage the tensile strength to resist the sheer forces exerted upon it during joint articulation (Klein et al., 2009).

Aggrecan molecules are held in place by this network of collagen fibres. As their negative charge acts to draw in water the tissue swells, this swelling pressure produces a tensile load in the cartilage fibres (Eckstein et al., 2001). It is this balance between the swelling pressure caused by the influx of water and the tensile resistance generated in the collagen fibres that allows cartilage to resist compressive loads (Eckstein et al., 2001). When cartilage experiences a load greater than the force generated by the swelling pressure, water is forced out of the tissue. This increases the concentration of proteoglycans and their large negative charge, draws water back into the tissue (James and Uhl, 2001). This movement of water out of the tissue under load, and its return during unloading, allows cartilage to act like a shock absorber, dissipating loads applied to it (van Osch et al., 1998).

Avascularity is a major reason behind the poor repair response of cartilage; this means that there is no supply of clotting materials or cells to produce repair material following damage or insult (Becerra et al., 2010). The avascularity may also mean that there is no supply of mesenchymal stem cells. These may be circulating cells or present as pericytes in the walls of blood vessels within in the tissue (Becerra et al., 2010). Evidence for poor blood supply being behind the reparative ability of cartilage can be seen when trauma or disease progress to a stage when bleeding into the defect can occur. Partial-thickness lesions are cartilage defects that do not penetrate the subchondral bone, lesions that do penetrate the subchondral bone plate are referred to as full-thickness lesions (Redman et al., 2005). Naturally occurring full-thickness lesions cause bone marrow blood to fill the defect forming a 'superclot', which leads to the development of a fibrocartilaginous repair tissue (Kim et al., 1991; Shapiro et al., 1993; Steadman et al., 2001).

Among the most common causes of damage to cartilage are trauma, osteoarthritis and osteochondritis dissecans (Madry et al., 2011). Each of these three conditions can present in similar ways, with pain, swelling and impaired movement of the joint (Madry et al., 2011). Treatment for these conditions is aimed at reducing pain and improving joint manipulation (Steinmeyer and Konttinen, 2006). As a result, the first line of treatments are non-steroidal anti-inflammatory drugs (NSAIDs), following these opiate pain killers may be used, some of the final options include hyaluronic acid or corticosteroid injections given into the joint (Steinmeyer and Konttinen, 2006). Once conservative medical options have been exhausted, there are a variety of different surgical options that can be used to treat damaged cartilage, these techniques are chosen due to patient factors such as age, body mass index (BMI) and activity levels, and factors based on the nature of the lesion e.g. size, type as well as location of the lesion within the joint (Michael et al., 2010).

The earliest surgical techniques to deal with damage cartilage were debridement and lavage; these were introduced in the 1940's and pioneered by surgeons like Magnuson and Haggart (Insall, 1967). The purpose of these procedures was to remove any loose cartilage bodies or fragments from the joint capsule, and to smooth any rough areas of cartilage, as these were at the time believed to be the cause of symptoms associated with cartilage damage (Insall, 1967). Debridement and lavage were originally performed as open techniques, they can now however be performed arthroscopically (Bird and Ring, 1978). These procedures have been shown to effectively reduce pain and increase joint mobility, as well as slow the progress of joint degeneration (Lutzner et al., 2009). However, Moseley et al. (2002) suggested that the improvements were simply a result of the placebo effect, as they found no difference between a sham surgery group and an experimental group.

Bone marrow stimulation techniques are based on the principle that when natural full depth defects cross the subchondral bone, bleeding from the bone marrow leads to the production of repair tissue within the lesion (Kim et al., 1991).

The earliest of these techniques was Pridie drilling, developed in the 1950's by KH Pridie (Insall, 1967).

This technique involves drilling holes into the subchondral plate with a drill (originally using a 1/4 inch drill bit) in order to stimulate bleeding (Insall, 1967).

Abrasion arthroplasty is a modification of the original 'housekeeping arthroplasty' technique and is similar to Pridie drilling but instead of using a drill bit an automated arthroscopic burr is used to perforate the subchondral bone to stimulate bleeding (Johnson, 1986). The third common marrow stimulation technique is microfracture, introduced by JR Steadman. This technique simply uses a surgical awl to create holes in the subchondral plate approximately 3-4 mm apart, which allow blood to enter the defect from the bone marrow bellow (Steadman et al., 2001).

The problem with these techniques is that although the clot produced fills the defect and produces repair tissue, this tissue is mostly fibrocartilaginous, and is a poor mechanical substitute for the natural hyaline cartilage (Chang et al., 2004; Kelly and Prendergast, 2005). The integration of the repair tissue has also been shown to be poor, which can lead to degeneration of the repair tissue or even necrosis (Mobasheri et al., 2009). Despite these problems, there has been some quite considerable success with this technique, particularly in younger more active patients (Madry et al., 2011). In rabbit models, there is even some evidence of the formation of more hyaline like repair tissue through the 'maturation' of a fibrocartilaginous tissue as a result of this technique (Steadman et al., 2001).

OATS (osteochondral transfer system) is a surgical repair procedure that involves the harvesting of osteochondral plugs from low weight-bearing regions of the joint or from an allogeneic or even cadaveric donor and placing them in to a the cartilage defect being repaired (Meyers et al., 1989; Hangody et al., 2004). There are two common forms of this procedure; osteochondral transfer (OCT) which involves the transfer of one plug from a harvest site to a recipient site, and mosaicplasty, which uses multiple plugs to fill larger recipient lesions (as large as 4 cm in diameter) (Hangody et al., 2004). This technique has been successful, resulting in decreased pain and improved mechanical function of the joint (Hangody et al., 2004). However, there is evidence of cell death at the donor site in autologous donors, this cell death leads to degeneration. Donor site degeneration can also occur as a result of repair tissue not forming naturally in response to bleeding from the subchondral plate triggered by plug removal, and may even require corrective surgery (LaPrade and Botker, 2004). The levels of integration seen in boney regions of the plug is good; however the cartilage regions have shown very little integration, which can cause to degeneration of the implant site, leading to graft failure (Chang et al., 2004).

Various other techniques have been used to repair damaged cartilage: osteotomies are used to correct malalignment of joints in order to prevent further degeneration due to abnormal loading (Wright et al., 2005). Soft tissue grafts of perichondrium or periosteum have also been transplanted in to cartilage defects to stimulate a repair response (Rubak, 1982; Amiel et al., 1985). The final solution for severe damage is a total joint replacement; however when joint replacement procedures are carried out in younger patients revision surgery becomes necessary, this is much more difficult than the initial surgery, and has a higher rate of complications (Klein et al., 2009). The most common treatment for OA in the USA is total joint replacement (Klein et al., 2009).

Cell based therapies

The difficulties associated with producing hyaline cartilage-like repair tissue in defects using these surgical techniques, combined with the problems associated with revision surgery of total joint replacements in young active individuals, has led to the expansion of tissue engineering approaches to treat damaged cartilage.

Tissue engineering is a very board term, and can cover a multitude of different approaches, strategies or mechanisms for the treatment of disease; however the classical view of tissue engineering is the use of scaffolds or matrices seeded *ex vivo* with cells to effect a repair in a target tissue (Bianco and Robey, 2001).

In order for cartilage therapy to be successful, either via surgical techniques or tissue engineering, the defect being repaired needs to be filled with a mechanically stable hyaline cartilage like substance that will not deteriorate over time and will integrate well with the surrounding tissue (Redman et al., 2005). Within the field of tissue engineering, there has been particular focus on the repair and regeneration of damaged cartilage. This wide-ranging interest has led to a number of different and varied approaches using different materials to fill cartilage lesions.

Currently, the only cell based tissue engineering approach that is licenced for use in patients is autologous chondrocyte implantation (ACI). This procedure, which was pioneered by Mats Brittberg and first used in 1987, involves the use of autologous chondrocytes, to produce repair tissue within a defect (Brittberg et al., 1994).

ACI involves two operations, in the first a small sample of cartilage (weighting between 200-300 mg) is removed from the joint periphery (such as the medial femoral condyle, or intercondylar notch). Once harvested the cartilage shavings are then enzymatically digested, first in pronase and then collagenase, to release the chondrocytes from the extra-cellular matrix (Brittberg et al., 1994; Brittberg, 2010). The chondrocytes are then expanded *in vitro*, care is taken not to allow the cells to expand too much, as if they undergo more than seven population doublings *in vitro* they begin to dedifferentiate, and so lose their reparative ability (Brittberg, 2010). Once the required numbers of chondrocytes have been produced they are reseeded back into the joint to affect a repair (Brittberg, 2010). The original ACI procedure involved harvesting a piece of perichondrium from the proximal medial tibia and

suturing the explant over the debrided defect, the flap was then sealed with fibrin glue and the chondrocytes injected underneath the flap (Brittberg et al., 1994). This initial technique had several problems e.g. the periosteal membrane could undergo hypertrophy causing the need for further surgery, the harvesting of the periosteum also introduced another site of morbidity alongside the cartilage harvest site and the defect itself. These problems lead to the second generation ACI technique, also referred to as collagen-covered ACI (CACI) (Brittberg, 2010). The replacement of the periosteal flap used in the first generation with a bilayer collagen membrane helps to prevent hypertrophy, and removes the morbidity associated with periosteal harvesting but the technique still requires an arthrotomy (Brittberg, 2010). The current third generation of ACI, called matrix-associated ACI (MACI) uses biodegradable collagen matrices seeded with the chondrocytes and anchored into the defect with fibrin glue (Brittberg, 2010). The advantage of this technique is that the first operation can be carried out with a miniarthrotomy, and the second arthroscopically, reducing the risk of complications as a result of or during surgery (Brittberg, 2010). ACI is most commonly used to treat larger defects (ranging from 3-10 cm^2), and has mainly been used within the knee joint (Madry et al., 2011).

ACI has been a successful technique, even the first generation used in the late 1980's produced improvements in joint function, reduced pain scores and in some cases hyaline-like repair tissue (Brittberg, 2010). Results of the efficacy of the first generation of ACI over several years have shown it to be an effective procedure; Micheli et al. (2001) found graft failure in only 6% of cases. Evidence also shows that when ACI is compared with microfracture surgery there is very little if any significant difference between the clinical outcomes of the two procedures (Knutsen et al., 2007). Despite the similarity in clinical outcome the histological quality of the repair tissue may be superior with ACI (Saris et al., 2008). MACI has been shown to produce 72% good or excellent results, but this was not significantly different from the 59% good or excellent results seen with the second generation CACI. However, the much improved surgical techniques used during the MACI make it a the preferable technique (Brittberg, 2010).

Despite this success, ACI has had and continues to have some problems. The first is the risk of donor site morbidity, this has been partially dealt with by moving away from the use of periosteal flaps, but still remains a problem at the cartilage harvest site. Damage to the cartilage can lead to further degeneration and can lead to osteoarthritis long term (Kim et al., 2008; Fischer et al., 2010). Another major drawback with ACI is the *in vitro* cell culture stage. When chondrocytes are cultured in a 2D environment for an extended period of time then they dedifferentiate. Dedifferentiation involves a decrease in the expression of collagen markers such as type II collagen and an increase in type I collagen production; the cells also develop a fibroblastic morphology (von der Mark et al., 1977; Weiss et al., 2010). This process prevents chondrocytes cultured for too long *in vitro* from being able to produce repair cartilage. The very low cell density in cartilage and the small areas available for harvesting, combined with their limited ability for useful expansion *in vitro*, means that ACI can only utilise a very small number of cells, where a larger number may have more success in producing hyaline like cartilage repair tissue (cell-cell contact is believed to be important for chondrogenesis) (Mueller and Tuan, 2008). Although ACI has been shown to produce hyaline like cartilage by Brittberg et al. (1994, 2010) and others questions have also been raised about the quality of ACI repair tissue, with other groups finding mechanically inferior fibrous tissue (Pelttari et al., 2008). ACI is also a multistep procedure, which increases the chances of an infection (especially during the earlier open approach) and increases the already high costs associated with the cell culture expansion stage (Mendelson et al., 2011).

There are a wide range of different approaches that are used to surgically treat damaged or diseased cartilage, ranging from marrow stimulation and debridement to cell based tissue engineering in the form of ACI. However, each of the aforementioned techniques has faults, as well as benefits, so other avenues are being explored to treat damaged cartilage. One of these avenues is the use of mesenchymal stem cells (MSCs) rather than chondrocytes in tissue engineering applications (Williams et al., 2010). This review describes MSCs and their potential uses in cell based tissue engineering based cartilage repair.

MSCs

Although interest in MSCs has increased exponentially over recent years they have been known about (by different names) for over 130 years (Abdallah and Kassem, 2008). In 1867, Julius Cohnheim proposed the idea that fibroblasts involved in wound repair may originate in the bone marrow. In the 1970's, Friedenstein and others like Owen showed that these fibroblast isolated by Cohnheim were multipotent cells capable of differentiating in to various forms of mesenchymal cells (Prockop, 1997; Chamberlain et al., 2007).

Dubbed mesenchymal stem cells by Arnold Caplan, these cells go by a number of names; pericytes, colony forming unit fibroblasts, mesenchymal stromal cells, but despite having a myriad of names there is not an absolute definition of what an MSC actually is (Fehrer and Lepperdinger, 2005). The broad definition of an 'MSC' is often given as; culture adherent multipotent progenitor cell that can differentiate down the adipogenic, chondrogenic and osteogenic lineages (Caplan, 2009a). This definition sounds conclusive, but the adherent cell cultures produce from MSC sources such as bone marrow are heterogeneous, so a more stringent definition is required (Battula et al., 2009). In order to refine this definition given by Caplan, others

have tried to use cell surface markers to provide a real distinction between MSCs and other adherent cells, however with the lack of a definitive MSC marker this has made this very difficult (Battula et al., 2009). Currently, the most quoted definition of an MSC comes from the International Society for Cellular Therapy (ISCT), their definition comes in three parts: In order to be considered a MSC a cell must be; plastic adherent, 95% or more of the cells of a colony must express CD105, CD73 and CD90, and less than 2% of the cells can express CD45, CD434, CD14, CD11b, CD79α, CD19 or HLA class II. Finally, they must be able to at least differentiate into adipocytes, chondrocytes and osteoblasts when stimulated *in vitro* (Dominici et al., 2006).

One of the roles of MSCs *in vivo* is the production of the bone marrow stroma (Panoskaltsis et al., 2005). The stroma is made up of the components of bone marrow that facilitate the haematopoietic process and the activity of haematopoietic stem cells and their progeny (Panoskaltsis et al., 2005). The stroma consists of an extracellular matrix which contains type I, III and IV collagen as wells as proteoglycans (Panoskaltsis et al., 2005). The matrix contains adipocytes, osteocytes and other cell types such as endothelial cells, which act together to create an environment suitable for haematopoiesis to occur (Panoskaltsis et al., 2005).

The original work on MSCs used cells isolated from bone marrow; we now know that MSCs can be found in a huge range of tissues including: fat, bone marrow, placenta, cartilage, foetal tissues (e.g. spleen and liver) and deciduous teeth (Bernardo et al., 2007). This plethora of different tissues from which we can isolate MSCs at various times of life poses an interesting question, where is the MSC's stem cell niche?

A stem cell niche is the region of a tissue or organ in which stem cells can be found, it provides much more than an anatomical location for the cells to reside in. The niche provides a microenvironment that allows them to function, particularly in adults (Scadden, 2006). Adult stem cells are believed to facilitate repair of damaged tissue, the niche allows stem cells to lie quiescent in adult tissues without being exposed to external factors that would cause differentiation and so depletion of the stem cell population (Scadden, 2006). The niche also provides the correct climate for the stem cells to self-renew, so when damage occurs the relevant stem cells can mount a reparative response, allowing effective repair without the depletion of the stem cell population (Scadden, 2006). It is believed that MSCs make up part of the population of pericytes (or mural cells) that surround blood vessels (Caplan, 2009b). Crisan et al. (2008) showed that a proportion of pericytes collected from a wide range of tissues could be selected by plastic adhesion and expanded long term in culture. The cells then demonstrated MSC like characteristics such as the ability to differentiate down the osteoblastic, chondrogenic and adipogenic lineages.

The stem cell niche is really a specific microenvironment, with cytokine levels, oxygen tension, mechanical stimuli and support structures that allow stem cell activity (Panoskaltsis et al., 2005). The stem cell niche, via signalling between cells and the surrounding matrix within the correct mechanical and biochemical conditions helps to regulate stem cell self-renewal and, at the same time, provides cells for the required differentiational lineages (Djouad et al., 2007). The niche can regulate the exposure of stem cells to certain signals; proteoglycans such as syndecan and glypican, which surround MSCs, are believed to bind to certain signalling molecules, preventing the ligands reaching naive MSCs (Djouad et al., 2007). Cross talk between cells and their environment plays a role in regulating the fate decisions of the cells, and is, therefore, important to understand from a therapeutic point of view (Djouad et al. 2007).

Built into the stem cell niche concept is the idea of the mechano-niche (Lee et al., 2011). Even subtle changes in the mechanical environment of a stem cell can, in a similar way to chemical changes, lead to a change in fate selection; these changes in load may result from changes such as alterations in the structure of the extracellular matrix (Lee et al., 2011). The mechanical stimuli that affect developing cells do not just come from loading of the tissue but also can be generated by the cells themselves, via cytoskeletal action on the surrounding matrix (Kelly and Jacobs, 2010).

As well as being found in many different tissue types, under the correct conditions MSCs are capable of producing a wide range of tissues *in vivo*. MSCs by definition can be differentiated into cartilage, bone and fat producing cells. They can also be differentiated into muscle, marrow and tendon as well as other types of mesenchymal tissue producing cells; including myeloid and lymphoid derived blood cells. There is even evidence of MSCs being used to produce non-mesenchymal cells such as hepatocytes and neurones, although these neuronal cells simply possessed a small number of neuronal cell markers, much more work is required to determine if MSCs can form functioning neurones (Mezey et al., 2000; Caplan, 2007; Wagner and Ho, 2007). It is the fact the MSCs can be differentiated in to a range of lineages *in vitro* that makes them such a potentially powerful tool within tissue engineering.

In order to differentiate MSCs down a desired lineage they must first be isolated from the original tissue sample. Within bone marrow there are only one to a hundred MSCs in every five thousand mononuclear cells, so selection is very important (Freyria and Mallein-Gerin, 2012). Two of the most common techniques for MSC isolation from bone marrow are adhesion to a tissue culture plastic, or density gradient centrifugation using ficoll (Baksh et al., 2003). Once isolated and proliferating in culture they can be directed to differentiate down various cell paths, using a range of soluble factors, or environmental/culture conditions (Chamberlain et al., 2007). Osteogenesis can be initiated in MSCs in monolayer by culturing them in a media

containing ascorbic acid, dexamethasone and ß-glycerophosphate (Chamberlain et al., 2007). This causes an increase in osteogenic markers such as alkaline phosphatase and, over time, calcium deposits form as nodules which can be detected with alizarin red or von Kossa stain respectively (Chamberlain et al., 2007). In a similar technique, adipogenesis can be initiated in monolayer MSCs using dexamethasone, insulin isobutyl methyl xanthine and indomethacin; this leads to the formation of lipid filled vacuoles within the MSCs, along with other adipogenic markers such as lipoprotein lipase and can be stained using the oil red O technique (Chamberlain et al., 2007). These lipid vacuoles coalesce and will ultimately fill the cell (Chamberlain et al., 2007). In order to induce chondrogenesis in MSCs, the cells need to be in close contact, as well as being exposed to the correct soluble factors (Mueller and Tuan, 2008). In order to achieve this MSCs are suspended in culture medium and spun in a centrifuge to produce a pellet culture (Johnstone et al., 1998). The pellet is then cultured in a growth medium containing TGF-ß, and leads to the development of cartilaginous tissue that stains for toluidine blue, and contains type II collagen (Johnstone et al., 1998). A problem with this cartilage model is, however, that TGF-ß induced chondrogenesis over time leads to hypertrophy of chondrogenic MSCs and an increased expression of osteogenic markers such as type X collagen and Runx2, in a similar progression of differentiation to that seen during bone formation via endochondral ossification, in which chondrocytes undergo hypertrophy leading to apoptosis calcification (Pelttari et al., 2008). The development of hypertrophy in MSCs that are induced down the chondrogenic lineage could potentially affect native tissue surrounding an MSC implant (Mueller et al., 2010). As a result, techniques to prevent hypertrophy need to be developed before chondrogenically induced MSCs can be used clinically.

When a tissue engineering technique is being developed, one of the most important factors to take into consideration is the type of cell to be transplanted in to the defect. In the case of cartilage, there are two candidate cell types, chondrocytes and MSCs. It will be important for future therapies to decide which of these two cell types is more appropriate for individual techniques, or even cartilage repair as a whole.

Chondrocytes are currently being used clinically in cartilage therapy as part of ACI. Although not directly being used for cartilage repair, MSC therapies are currently being developed for example the use of MSC in the repair of large bone defects as well as in non-skeletal conditions such as graft vs. host disease and to repair cardiac muscle after myocardial infarction (Chamberlain et al., 2007; Caplan, 2009; Mobasheri et al., 2009).

The current method for chondrocyte collection in ACI involves the removal of slithers of cartilage, from which chondrocytes can be extracted; these slivers are collected form the joint during a miniarthrotomy which involves partial opening of the joint cavity (Brittberg, 2010). MSCs can be harvested from the bone marrow of the iliac crest using a syringe, a procedure called a bone marrow aspiration (Weiss et al., 2010). Although a bone marrow aspiration is painful for the patient, this can be overcome with local anaesthesia. It involves far less risk that the miniarthrotomy which may expose the joint cavity to infection, and has the same risks as any other procedure that involves general anaesthesia (Weiss et al., 2010). The collection of cartilage from the surface of the joint can also lead to degeneration and increases the long-term risk of osteoarthritis (Fischer et al., 2010).

When removed from their natural 3D environment in the extracellular matrix of articular cartilage, chondrocytes lose any potentially important stimulation from mechanical forces or soluble factors (e.g. growth factors) that exist within the tissue. As a result, culturing chondrocytes in monolayer causes them to undergo dedifferentiation (Bosnakovski et al., 2006; Freyria and Mallein-Gerin, 2012). Another factor in the progression of dedifferentiation is the loss of the chondrocytes natural spherical morphology, which cannot be maintained in monolayer culture, this results in the adoption of a flattened fibroblast like morphology (Benya and Shaffer, 1982) Dedifferentiation can be prevented by culturing cells in an environment in which they cannot lose their spherical morphology e.g. in in suspension culture or within an agarose gel (Benya and Shaffer, 1982). These changes reduce the chondrocytes chondrogenic ability and causes an increase in markers such as type I collagen (Freyria and Mallein-Gerin, 2012). On the other hand, MSCs maintain their chondrogenic ability, even throughout long term monolayer culture, although their replicative capacity is not infinite. Friedenstein et al., (1987) could produce differentiation in cells that had undergone thirty population doublings. Whilst Banfi et al. (2000) estimated that the useful clinical limit for expansion would be seventeen population doublings, more than twice as many as chondrocytes can be usefully expanded by. As the number of population doublings that an MSC culture has been through increases, the proportion of larger cells in the population begins to increase. These cells are less active, and have less differentiation capacity than the smaller rapidly self-renewing cells in the population (RS cells) (Smith et al., 2004; Fehrer and Lepperdinger, 2005). Along with the increase in larger cells, individual cells lose their ability to differentiate in to adipocytes, chondrocytes and osteoblasts in a stochastic (hierarchal manner) (Muraglia et al., 2000). The first lineage that is closed to MSCs is adipogenesis. This occurs at approximately twenty two population doublings, the cells then retain the ability to form chondrocytes and osteoblasts for a considerable time in culture before chondrogenesis is lost and the cells can only become osteoblasts (Muraglia et al., 2000). As well as maintaining their ability to produce useful cartilage-like repair tissue longer than chondrocytes in culture,

MSCs also have a higher rate of proliferation, and unlike chondrocytes, this proliferative rate does not decrease when the cells are cultured in monolayer or when they are harvested from an older donor (Steck et al., 2009; Bosetti et al., 2012).

When chondrocytes are grown in high density pellet cultures they are more effective than MSCs at producing and maintaining cartilage like tissue. The tissue produced by chondrocytes is also mechanically superior and contains higher levels of aggrecan and type II collagen (Bernstein et al., 2010). When cultured in pellets, chondrocytes maintain their phenotype, however when MSCs are induced down the chondrogenic lineage in a pellet culture their differentiation does not stop with the chondrocyte phenotype, but they rapidly enter hypertrophy and begin to express osteogenic markers e.g. type X collagen and MMP-13 (Barry et al., 2001). This difference suggests that the differentiation that MSCs undergo when TGF-ß is used to induce chondrogenesis is closer to the differentiation seen in the growth plate than that seen at the distal surfaces of long bones where stable chondrocytes form (Mueller and Tuan, 2008). Identifying the differences between these two processes may allow for improvement of MSC differentiation protocols (Pelttari et al., 2006). When pellet cultures of chondrocytes are implanted in to the subcutaneous pouch of severe combined immune deficiency (SCID) mice they remain as stable ectopic pieces of cartilage. When the same experiment is carried out with pellets of MSCs induced into the chondrogenic pathway neovascularisation and calcification occur (Pelttari et al., 2006). This may be due to the fact that MSCs are not undergoing ordered chondrogenesis, but individual cells in the heterogeneous population may be expressing different phenotypic markers (Pelttari et al., 2006). This disorder may prevent MSCs from maintaining the chondrogenic phenotype in culture (Pelttari et al., 2006). The withdrawal of TGF-ß from the pellet may also trigger the hypertrophic response rather than maintaining the chondrogenic phenotype seen before implantation (Mueller et al., 2010). The heterogeneous nature of MSC populations means that individual cells will have different capabilities to proliferate and differentiate down different pathways, which may add to the disorganisation seen pellet cultures (Mueller and Tuan, 2008).

The apparent predisposition of bone marrow MSCs to progress towards chondrogenesis may also be linked to their role within bone tissue. MSCs play an important role in bone healing after fracture (Dimitriou et al., 2005). These cells act as a key source of stem cells, which play a critical role in various stages of the fracture healing process (Dimitriou et al., 2005). This may mean that the cells harvested form bone marrow may have some underlying predisposition to osteogenesis rather than chondrogenesis.

When grown in monolayer culture chondrocytes down regulate chondrogenic gene expression, but they do not progress through terminal differentiation or hypertrophy, even in the presence of TGF-ß. This is further evidence that the chondrogenic differentiation seen in MSCs is different to that seen in articular chondrocytes, despite similarities in matrix production and behaviour (Pelttari et al., 2006).

Interestingly, a population of progenitor cells derived from the cartilage tissue itself have shown the propensity to undergo a large number of population doublings, while not losing its chondrogenic potential (Dowthwaite et al., 2004; Khan et al., 2009). These cells where first described by Dowthwaite et al. (2004), they are harvested from the surface zone of articular cartilage using fibronectin adhesion. Further characterisation of these cells has shown that these chondroprogenitors maintain their chondrogenic ability over the course of monolayer culture and can be induced into chondrogenesis in pellet culture even at thirty population doublings (Khan et al., 2009). These progenitors have also been shown to maintain the expression of the stem cell markers STRO-1, NOTCH-1 and CD 90 after thirty population doublings in monolayer culture (Williams et al., 2010). The telomerase activity of this population of cells is higher at both at low (3.1 fold higher) and high (10.3 fold higher) population doublings when compared to full depth chondrocytes (Williams et al., 2010).

These cells can undergo monolayer expansion and without losing the ability to undergo chondrogenesis, without showing a decrease in the expression of stem cell markers or a decrease in telomerase activity (Khan et al., 2009; Williams et al., 2010). These features may mean that surface zone chondroprogenitors do no suffer the same problems of terminal differentiation and hypertrophy that we see in MSCs isolated from bone marrow or synovial membrane in chondrogenic conditions (De Bari et al., 2001; Kafienah et al., 2007). This makes them extremely attractive for tissue engineering applications. Their amenity to monolayer culture also may allow the use of fewer cells to treat larger lesions by using an *in vivo* expansion technique. This would minimise the donor sight morbidity seen in ACI currently.

As these cells do not show signs of hypertrophy, they offer an alternative source of progenitor cells with a more stable phenotype for cell based therapeutic techniques.

Due to their good proliferative capacity, multipotent differentiation capacity and availability in potentially high numbers from a range of tissues MSCs are extremely attractive for use in tissue engineering. However more understanding is needed on various soluble factors such as growth factors and other signalling molecules which guide and control the differentiation MSCs before they will be useable *in vivo* (Pelttari et al., 2008; Weiss et al., 2010).

Soluble factors

There are a huge number of cytokines, hormones, growth factors and other signalling molecules that affect

the action of chondrocytes within developing and adult cartilage. These factors can both promote cartilage production and a chondrogenic phenotype (e.g. IGF-1 or PTHrP) as well as cause cartilage degradation or turnover of cartilage to form bone (e.g. IHH) (Deckelbaum et al., 2002; Kobayashi et al., 2005; Davies et al., 2008). This review will focus on four key soluble signalling factors; the two cytokine growth factors IGF and TGF-ß, and the axis formed between IHH and PTHrP.

Transforming growth factor-ß

The name transforming growth factor ß (TGF-ß) refers to a super-family of cytokines rather than a single specific factor (Bosetti et al., 2012). This superfamily includes three forms of TGF-ß (TGF-ß1, 2 and 3), and other factors such as bone morphogenetic proteins (BMPs) (Huang et al., 2004; Mackie et al., 2011). Despite being members of the same family the individual factors have different effects e.g. TGF-ß1 and -3 cause chondrogenesis in MSCs, whereas TGF-ß2 has been shown to be chondrogenic by Barry et al. (2001) and van Osch et al. (1998) but not by Bosetti et al. (2012). It has been demonstrated that the chondro-inductive effects of both TGF-ß1 and TGF-ß3 on MSCs in chondrogenic conditions are extremely similar (Mueller et al., 2010). Members of the TGF-ß super-family are expressed by cells in all stages of chondrogenesis, and the family is believed to have an important role in chondrogenesis during developmental processes such as endochondral ossification (Longobardi et al., 2006). Members of the TGF-ß family are some of the first factors to be expressed during the initial condensation of mesenchymal cells during endochondral ossification, TGF-ßs are also important for triggering chondrocyte proliferation later in the process (Goldring et al., 2006).

Current interest surrounding TGF-ß within the field of cartilage tissue engineering is based on the ability of TGF-ß1 to induce chondrogenesis in chondrocytes and MSCs grown *in vitro* (Johnstone et al., 1998; Diekman et al., 2010). TGF-ß on its own can induce chondrogenesis in cells in culture, however this effect is enhanced by the presence of factors such as insulin-like growth factor (IGF-1), dexamethasone (which has been shown to aid terminal chondrogenic differentiation) and BMP-6 (which does not induce chondrogenesis on its own) (Johnstone et al., 1998). These supplementary factors facilitate and enhanced the chondrogenic effects of TGF-ß (Indrawattana et al., 2004; Takagi et al., 2007). For this reason, so called 'chondrogenic medium' contains not only TGF-ß-1 but also supplementary factors like dexamethasone to enhance the chondrogenic effects of TGF-ß (Li et al., 2010a). When exposed to a chemically defined chondrogenic medium in culture, MSCs undergo an increase in proliferation as well as an increase in the expression of chondrogenic markers characteristic of primary chondrocytes (Longobardi et al., 2006). These markers include the master chondrogenic regulating transcription factor SOX9, and key cartilage matrix components such as type II collagen and aggrecan (Longobardi et al., 2006; Pelttari et al., 2006). This leads to the deposition of a proteoglycan rich matrix around the cells (Van Osch, 1998). TGF-ß is also important for the expression of glycosaminoglycans (GAG). Kupcsik et.al. (2010) found that even in the presence of mechanical loading TGF-ß1 concentration was responsible for 60% of the variation of GAG production, TGF-ß has also been linked to increased GAG production in bovine articular chondrocytes (Davies et al., 2008).

As well as causing increases in chondrogenic markers, TGF-ß has also been shown to down-regulate PRG4 expression in hMSCs. This gene is responsible for the production of surface zone protein (SZP) (Li et al., 2010b). This proteoglycan is crucial in providing lubrication at the joints articulating interface (Ng et al., 2009). However, SZPs effects appear to be further reaching than lubrication. Mice with the PRG4 gene knocked out suffered joint failure as a result of degeneration of the cartilage surface layer and synoviocyte over-expansion, which could be treated by the administration of the SZP analogue lubricin (Ng et al., 2009).

Thorpe et al. (2010) showed that TGF-ß could enhance the chondrogenesis induced in MSCs by mechanical load, if they were first precultured in a TGF-ß containing media. Constructs precultured with TGF-ß demonstrated enhanced GAG retention, and those continuously exposed to TGF-ß showed better retention than those only transiently exposed (Thorpe et al., 2010). This increased matrix production lead to an improvement in the quality of those constructs treated with TGF-ß3 (Thorpe et al., 2010).

As well as these direct chondrogenic effects on MSCs and chondrocytes, there is also evidence for TGF-ß3 possessing chemotactic effects (Mendelson et al., 2011). Mendelson et al. (2011) showed that TGF-ß3 could be used *in vivo* to induce the migration of stem cells found in tissues around the joint, for example endochondral stem cells, and MSCs where shown to migrate into collagen sponges containing TGF-ß3 (Mendelson et al., 2011). The potential ability to recruit cells capable of initiating a repair from neighbouring tissues would provide an extra advantage to a cellular technique using TGF-ß within a scaffold.

Members of the TGF-ß superfamily form dimeric ligands, and initiate the relevant signal cascades via two serine/threonine receptors, the TGF-ß type I and II receptors (Derynck and Zhang, 2003). The first step in the signalling cascade is the binding of the dimeric ligand to the high affinity TGF-ß type II receptor. This activates the kinase activity of the type II receptor to phosphorylate the low affinity type I receptor (Derynck and Zhang, 2003; Longobardi et al., 2006). The activation of the type II receptor after phosphorylation leads to the formation of a complex between the two

receptors called the TGFßRII- TGFßRI complex (Longobardi et al., 2006). The TGFßRII- TGFßRI receptor complex then activates the R-smad cascade by phosphorylating receptor-Smads, which then move to the nucleus and triggers the stimulation of TGF-ß target genes via interaction with zinc finger transcription factors called Schnurri proteins (Longobardi et al., 2006). There is also evidence for the existence of Smad independent signalling, e.g. via the MEK1/2-ERK1/2 pathway (Longobardi et al., 2006).

The ability of TGF-ß1, -ß3 to induce chondrogenesis in MSCs *in vitro* makes it an extremely valuable component in cartilage tissue engineering. However, as well as inducing chondrogenic factors TGF-ß also causes type X collagen expression when given to cells *in vitro* (Kafienah et al., 2007). Type X collagen is a marker of hypertrophy, and when given to MSCs *in vitro* (but not chondrocytes) TGF-ß causes hypertrophy, this prevents the cells from being therapeutically useful (Kafienah et al., 2007). It may be that other soluble factors that aid and influence TGF-ßs action such as IGF or PTHrP this may allow for the artificial prevention of hypertrophy in MSCs induced into chondrogenesis by TGF-ß.

The extent of TGF-ß's effects may extend past chondrocytes to cartilage tissue itself. Khan et al. (2011) showed that culturing explants of immature bovine cartilage in the presence of TGF-ß and FGF caused an accelerated maturation of the tissue *in vitro*. This work demonstrated a decrease in explant size, and an increase in their compressive modulus (Khan et al., 2011). There were also other signs of cartilage maturation for example; increases in mature collagen crosslinks compared to higher degrees of immature crosslinks in control explants (Khan et al., 2011). This enhanced maturation of cartilage tissue could be used to aid the post treatment development of hyaline like repair tissue in surgically treated cartilage defects.

Load and endogenous TGF-ß production

It has been repeatedly shown that exposing MSCs and chondrocytes to various forms of load can lead to the up regulation of chondrogenic genes, or even elicit chondrogenic responses in cells that are not exposed to the highly chondrogenic cytokine TGF-ß. The effects of loading are very specific to individual areas within the musculo-skeletal system; it is, therefore, probable that it is the role of locally acting factors such as cytokines with paracrine signalling roles that mediate the effects of mechanical loading (Klein-Nulend et al., 1995). It has been suggested by several groups since the mid nineteen-nineties that loading stimulates the endogenous production of TGF-ß in MSCs and chondrocytes, which then much like the exogenous TGF-ß in chondrogenic media, stimulates chondrogenic changes within cells (Klein-Nulend et al., 1995; Huang et al., 2004; Li et al., 2010b).

Klein-Nulend et al. (1995) showed that mouse calvariae and periosteal cells produced TGF-ß in response to hydrostatic pressure applied via the gas phase at 0.3 Hz and a maximum pressure of 13kPa, for ten days. The authors also noted that the endogenous production of TGF-ß in response to loading had the same effects on cellular activity as exogenous TGF-ß added to the culture medium (Klein-Nulend et al., 1995). The effects of cyclic compression on TGF-ß have been demonstrated on MSCs harvested from the bone marrow of rabbits and implanted in to 2% agarose (Huang et al., 2004). Cells in experimental groups were exposed to 10% strain at 1Hz for four hours a day (Huang et al., 2004) The loading of cells in the presence and absence of TGF-ß and the effect of TGF-ß on unloaded cells was documented as part of this study (Huang et al., 2004). The results showed that there was a significant increase in the levels of aggrecan and type II collagen expression between the loaded cells that were exposed to TGF-ß and those that were not, cells exposed to TGF-ß but not loaded and the control group which were not loaded or exposed to TGF-ß (Huang et al., 2004). The combined effect of TGF-ß and loading was an increase in the production of type II collagen when compared to the cells exposed to TGF-ß alone (Huang et al., 2004). The similarity in the effects of loading alone and TGF-ß alone suggest that there may be links in the mechanisms of the two systems (Huang et al., 2004). This study also showed that the loading of cells increased the expression of TGF-ß in the experimental groups compared to the control groups (Huang et al., 2004).

In a follow-up to their 2004 study Huang et al. published further research on the effects of loading on TGF-ß production in 2005. This study used a slightly altered experimental approach with cells exposed to 15% loading for four hours at 1Hz for two consecutive days (Huang et al., 2005). This study was designed to determine the temporal production characteristics of various factors associated with the early stages of endogenous TGF-ß signalling in loaded rabbit bone marrow MSCs (Huang et al., 2005). The results show up-regulation of both parts of the dimeric TGF-ß receptor, *TßR-I* and *TßR-II*, as well as the up regulation of the *c-Jun* and *c-Fos* genes, which are associated with the ERK and MAPK pathways, which are associated with TGF-ß signalling (Huang et al., 2005). The master chondrogenic transcription factor SOX 9 was also found to be up-regulated in loaded cells (Huang et al., 2005).

In applying a combination of shear and compression to MSCs, Li et al. (2010b) demonstrated that the beneficial effects of loading on the expression of chondrogenic genes were negated it the cells were exposed to TGF-ß within the culture medium. In order to establish the link between loading and production of endogenous TGF-ß, MSCs were cultured in a medium containing the TßR-I blocker LY364947 (Li et al., 2010b). The effect of this blocker was to inhibit the up regulation of TGF-ß -1 and -3 that is seen in MSCs exposed to load (Li et al., 2010b). It has since been demonstrated the shear is critical in producing this

response (Fig. 1) (Schätti et al., 2011).

The evidence provided by these investigations, clearly show the link between the loading of cells *in vitro* and the up-regulation of TGF-ß1 and other genes associated with TGF-ß and its signalling. The expression of this endogenous TGF-ß then produces the expression of chondrogenic genes that is desired in loaded cells.

Insulin like growth factor-1

Insulin like growth factors (IGFs), like members of the TGF-ß family are important signalling molecules in both development and adult life (Longobardi et al., 2006). Mice with the gene for IGF-1 (*Igf*) knocked out suffer from growth failure, reaching approximately 60% of the normal birth weight, some may reach adulthood but others die shortly after birth (Liu et al., 1993). As well as being important in development, IGF is also one of the main anabolic factors within cartilage (Davies et al., 2008). IGF does not only induce the expression of matrix molecules like type II collagen and proteoglycans, but also decreases the activity of matrix metalloproteinases by increasing the expression of tissue inhibitors of metalloproteinases (TIMPS) (Davies et al., 2008). IGF is found free in the synovial fluid (up to 50 ng/ml) and in the cartilage extracellular matrix (10 ng/ml) (Davies et al., 2008). When administered to cells *in vitro* IGF appears to produce a positive feedback loop, causing an increase in the production of IGF mRNA by mature bovine chondrocytes. IGF-1 also produces an increase in the production of IGF receptor (IGF1R) mRNA and, in doing so, up-regulating matrix biosynthesis (Davies et al., 2008). This may also partially explain the decreased expression of matrix products with increased age due to a decrease in the expression of IGF-1R (Davies et al., 2008). It has also been shown to increase the retention of synthesised matrix (van Osch et al., 1998).

One of IGFs most important roles during growth is the mediation of growth hormone signalling at the growth plate (Mackie et al., 2011). The action of growth hormone, which is produced in the pituitary, is mediated by the synthesis and release of IGF by both liver cells and chondrocytes (Mackie et al., 2011). This mechanism is referred to as the GH/IGF axis and is crucial for the correct regulation of bone growth (Mackie et al., 2011).

IGF-1 signals via a tyrosine kinase receptor called IGF-1R. Once IGF binds to this receptor it initiates a signalling cascade by phosphorylating tyrosine residues on molecules in the MEK1/2, Erk1/2, PI3K-Akt and the MAPK pathways, leading to the expression of IGF target genes (Longobardi et al., 2006). Both IGF-1 and IGF-2 which are both found in the growth plate act through the IGF-1R (Mackie et al., 2011).

As previously mentioned, the consensus view is that IGF alone cannot induce chondrogenesis in MSCs; however this has been called into question by Longobardi et.al. (2006) who showed that when cells were cultured in the absence of the ITS (insulin, transferrin and selenium) in the medium, IGF-1 could induce proliferation, decrease apoptosis and the expression of chondrogenic markers. This is due to the antagonistic effects of non-specific insulin stimulation of the IGF-1R (Longobardi et al., 2006). The group also showed that the two growth factors act via different mechanisms as they acted synergistically when given

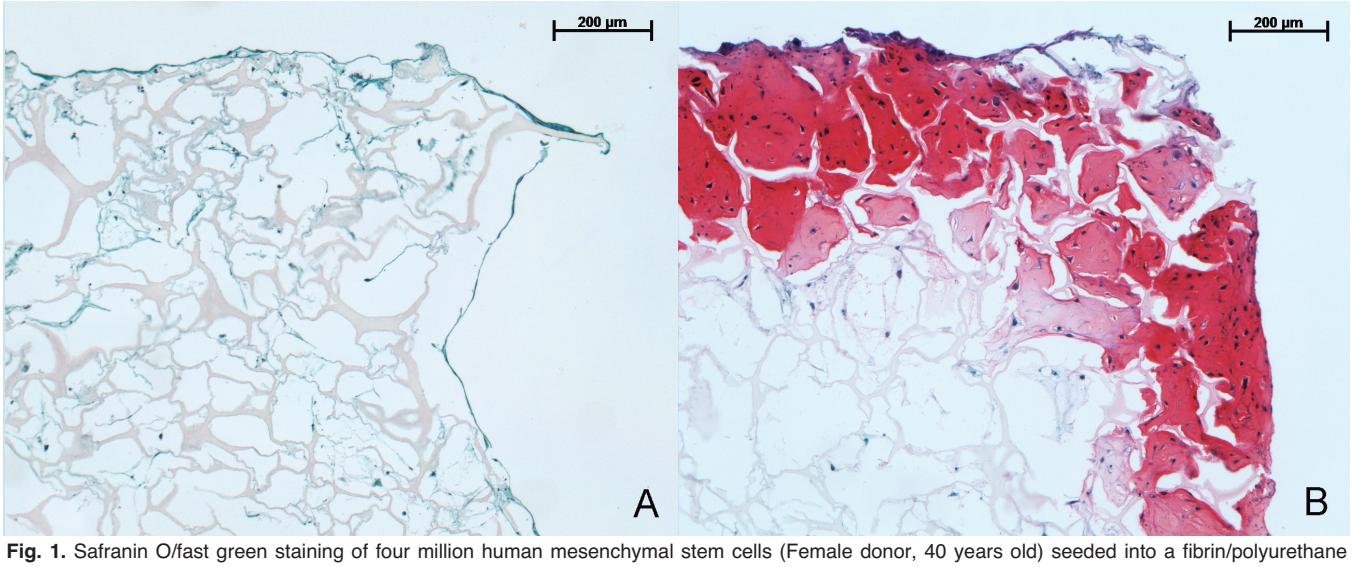

Fig. 1. Safranin O/fast green staining of four million human mesenchymal stem cells (Female donor, 40 years old) seeded into a fibrin/polyurethane composite scaffold after 28 days of culture. **A.** Under free swelling conditions in the absence of exogenous TGFß. **B.** With compression and shear applied 1 hour a day in the absence of exogenous TGFß. As can be seen, the loaded group stains intensely orange, indicating the presence of abundant glycosaminoglycan. This is not seen in the unloaded group. Bar: 200 µm.

simultaneously to proliferating cells (Longobardi et al., 2006). This is an example of how the understanding of growth factors and their actions still requires further investigation.

IGF is already being considered as a tool within tissue engineering. Fortier et al. (2002) incorporated IGF-1 into fibrin scaffolds along with chondrocytes and implanted these in to large full thickness defects in horses. The grafts containing IGF showed better integration with natural tissue, better filling of the defect and reduced fibrillation (Fortier et al., 2002). There was also an up regulation of matrix components such as hyaluronan, type II collagen as well as less proteoglycan degradation, resulting in higher quality repair material (Fortier et al., 2002).

This is not the only example of growth factors being used *in vivo* in a tissue engineering approach, Madry et.al. (2010) produced transfected IGF-1 and FGF-2 expressing 3T3 fibroblasts using a DNA transfection technique. These cells were then implanted within an alginate hydrogel matrix in to full depth defects of the rabbit trochlear groove. This approach led to an increase in DNA within the graft as a result of proliferation as well as an increase in type II collagen and proteoglycan expression, which allowed for accelerated and enhanced tissue repair when compared to controls (Madry et al., 2010).

Indian hedgehog

Indian hedgehog is a member of the hedgehog family; this is a group of secreted morphogens found in vertebrates (Deckelbaum et al., 2002). The three members of the hedgehog family are desert hedgehog (DHH), sonic hedgehog (SHH) and Indian hedgehog (IHH) (Lai and Mitchell, 2005). DHH is involved in the formation of peripheral nerves, SHH is important in lateral-asymmetry, and IHH is the main regulator of bone development via endochondral ossification, as well as playing important roles in the kidney and gastrointestinal tract (Lai and Mitchell, 2005). The hedgehog signalling molecules are formed through the autocatalysis of a parent protein, whose catalytic C-terminal domain releases its N-terminal domain as the secreted hedgehog peptide (Deckelbaum et al., 2002). IHH is produced in the growth plate by prehypertrophic and hypertrophic chondrocytes (Kobayashi et al., 2005). It acts in a paracrine manner on the cells of the perichondrium, which it stimulates to produce PTHrP; IHH also triggers the proliferation, maturation and terminal differentiation and eventual hypertrophy of growth plate chondrocytes (Deckelbaum et al., 2002; Kobayashi et al., 2002). The proliferative increase caused in chondrocytes occurs through the up-regulation of proliferative genes following the phosphorylation and activation of E2F transcription factors by cyclin dependent kinases, which are activated by IHH dependent increases in cyclin D1 (Mackie et al., 2011). Over-expression of IHH induced using retroviral transfection into chondrocytes shows that increased IHH expression leads to an increase in the chondrogenic marker type II collagen and the hypertrophic markers type X collagen and alkaline phosphatase (ALP) (Deckelbaum et al., 2002). However, this research also showed that in IHH-null mice, the growth plate chondrocytes still progressed to hypertrophy, but at a slower rate than wild-type mice, demonstrating that IHH is not the sole driver of chondrocyte differentiation in the growth plate (Deckelbaum et al., 2002).

IHH signalling begins when IHH binds to a 12-trans membrane domain protein receptor called patched-1 (Ptc), this binding causes the down-regulation of the inhibition exerted in the absence of IHH by Ptc on another membrane associated 7-transmembrane domain protein called smoothened (Smo). Once activated, Smo initiates the expression of IHH target genes such as ALP and type X collagen via the Gli family of transcription factors (Deckelbaum et al., 2002; Kobayashi et al., 2005; Mackie, 2011). The Gli transcription factors are three homologues of the Cubitus interuptus (Ci) zinc finger, which can act as repressors or activators (Lai and Mitchell, 2005). In the presence of IHH, the repressor Gli3 is down-regulated whilst the activator Gli1 is up-regulated (Lai and Mitchell, 2005).

IHH signalling is regulated in a number of ways. IHH is subject to spatial regulation from heparan sulphate proteoglycans, which appear to limit the movement of IHH to within the growth plate (Lai and Mitchell, 2005). This mechanism may not be designed to limit the spread of IHH, but rather to target the IHH towards proliferating chondrocytes which express specific proteoglycans (Lai and Mitchell, 2005). The main source of regulation for IHH comes from parathyroid hormone-related peptide (PTHrP) (Lai and Mitchell, 2005). IHH and PTHrP form a negative feedback loop that is responsible for the regulation of bone development through the process of endochondral ossification, and also the lengthening of long bones at the epiphyseal growth plates (Mackie et al., 2011).

Parathyroid hormone related protein

Parathyroid hormone related protein (PTHrP) is produced in the periarticular chondrocytes and perichondrial cells in response to IHH stimulation (Mau et al., 2007). There are three proposed mechanisms by which IHH may up-regulate the expression of PTHrP; the first is that IHH directly stimulates the cells of the periarticular perichondrium to secrete PTHrP. The second and third involve the indirect stimulation of PTHrP release via either BMP2/4 or TGF-ß2 as intermediate signalling molecules, the degrees of importance of these individual systems is not currently known (Lai and Mitchell, 2005). PTHrP acts to prevent the differentiation of chondrocytes or MSCs towards terminal differentiation and hypertrophy that is induced IHH; PTHrP helps to preserve the chondrogenic phenotype within the developing growth plate by

interfering with IHH signalling pathways (Kim et al., 2008; Fischer et al., 2010). The over-expression of the PTHrP receptor, expression of a constitutively active receptor or the over-expression of PTHrP have shown to cause a slowing in the rate of chondrocyte hypertrophy compared to controls (Rabie et al., 2003). Kobayashi et al. (2002) showed that PTHrP and PTHrP receptor null-mice rapidly developed chondrodysplasia due to the rapid and uncontrolled hypertrophy within the growth plate. These expression studies and the clear effects of the under- or over-expression of PTHrP demonstrate the importance of PTHrP as a regulator of IHH.

It is PTHrP's ability to maintain the chondrogenic phenotype of resting zone chondrocytes, despite IHH signalling acting to induce hypertrophic differentiation, which makes PTHrP interesting within tissue engineering. This effect may allow PTHrP to be used to preventing hypertrophy in MSCs induced into chondrogenesis using TGF-ß and, therefore enable, their use in clinical procedures.

PTHrP exerts its effects on IHH through the seven transmembrane G-protein couple receptor PTHR1; PTHrP binding causes the activation of a range of signalling cascades e.g. PKA, PKC and IP3 (Mau et al., 2007; Kim et al., 2008). One effect of the PKA pathway is to maintain the presence of the Gli3 transcription factor, as already mentioned is a negative regulator for IHH target genes, so preventing its down regulation in the presence of IHH allows PTHrP to inhibit the terminal differentiation of chondrocytes that is driven by IHH (Mau et al., 2007).

As well as inhibiting hypertrophy, PTHrP also increases the proliferation rate of MSCs cultured in a TGF-ß containing chondrogenic media. PTHrP does this by supressing the P57 gene, which is associated with quiescence in cells that express it (Kim et al., 2008). PTHrP also leads to an up-regulation of the chondrogenic markers type II collagen and SOX9, in a dose dependent fashion. There was also a decrease in the marker of hypertrophy type X collagen, and a PKC related decrease in the expression of Runx2, another hypertrophic marker (Kim et al., 2008).

PTHrP appears to affect chondrocytes (and MSCs) induced down the chondrogenic lineage at particular time points within their development (be this due to the exogenous environment, or endogenous processes) namely early and late (Rabie et al., 2003). If applied early in differentiation, the PTHrP will inhibit chondrogenesis, it will also prevent any further progression down the chondrogenic lineage in the later stages of differentiation, and down-regulating the expression of hypertrophic markers (as the cell progresses towards hypertrophy) (Weiss et al., 2010). If, however, PTHrP is applied at an intermediate time point, PTHrP will enhance the proliferation of the cells and the expression of chondrogenic genes in chondrocytes and support the differentiation of MSCs into chondroblasts (Rabie et al., 2003).

The beneficial effects of PTHrP on chondrogenic cells are only maintained as long as the cells are in the presence of the growth factor; this was demonstrated by Weiss et al. (2010) who implanted MSCs in micromass pellet cultures into SCID mice, in the absence of further PTHrP stimulation the pellets underwent vascular invasion and ossification.

PTHrP-IHH Axis

Within the cartilage growth-plate, it is not the individual effects of PTHrP and IHH that are important, it is the effects that they generate together as a feedback loop that sets them apart. The relationship of PTHrP and IHH was first described by Vortkamp et al. (1996), the feedback loop that they form regulates initial formation of bone via endochondral ossification and after that the lengthening of bone through the activity of the growth plate (Fig. 2). This is achieved through the regulation of chondrocyte differentiation (Lai and Mitchell, 2005). In

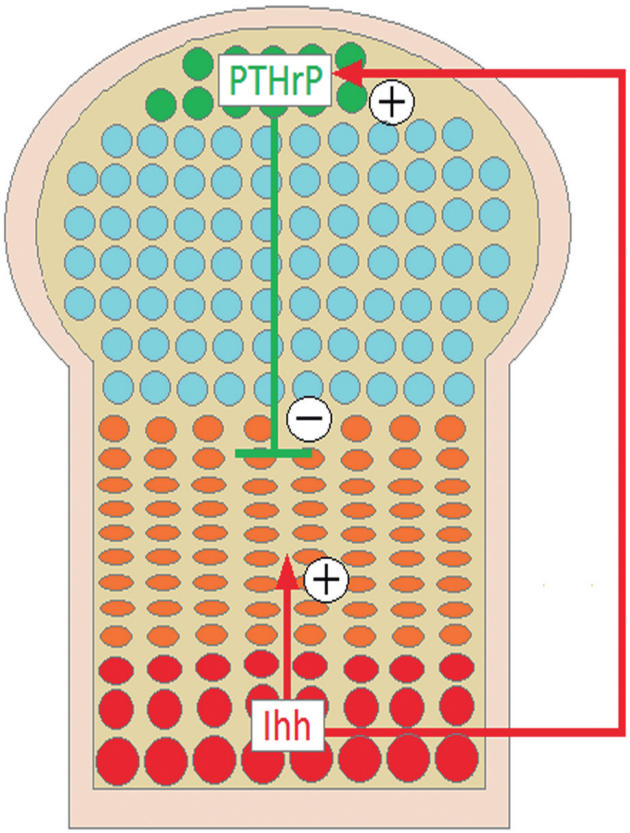

Fig. 2. The epiphyseal growth plate, showing the PTHrP producing peri-articular chondrocytes in green, resting zone chondrocytes in blue, proliferating zone cells in orange and Ihh producing hypertrophic cells in red. Arrows indicate the PTHrP-Ihh axis which controls the rate of growth plate development by regulating the progression of chondrocytes towards terminal differentiation; from resting zone chondrocytes to hypertrophic cells.

order to demonstrate the action of this mechanism, this review will focus on growth plate elongation.

The growth plate is a band of hyaline cartilage that sits in the metaphysis of long bones; it has a clearly defined structure that allows the elongation of the epiphyseal aspect of the plate, and the calcification of the diaphyseal aspect of the plate, resulting in elongation of the bone.

The growth plate consists of several layers of chondrocytes between the secondary and primary centres of ossification the periarticular cells and resting chondrocytes (which sit adjacent to secondary centre of ossification), the proliferating zone, the prehypertrophic zone, and the hypertrophic zone (adjacent to the primary centre of ossification) (Mackie et al., 2011).

IHH, as already mentioned is produced by hypertrophic chondrocytes of including those cells entering hypertrophy in the prehypertrophic and hypertrophic zones of the growth plate, this signalling acts to increases the number of chondrocytes in the proliferating zone entering terminal differentiation (Kronenberg, 2003; Lai and Mitchell, 2005). The expression of IHH by these cells also leads to the expression of PTHrP from the periarticular cells (Kronenberg, 2003). The PTHrP, in turn, prevents the proliferating chondrocytes from entering terminal differentiation by up-regulating the expression of chondrogenic genes, and blocking the action of IHH signalling by binding to PTHR1 and up-regulating the expression of Gli3, forming a negative feedback loop (Kronenberg, 2003; Mau et al., 2007). The action of these two hormones controls the length of the growth plate by regulating the speed of chondrogenesis in the proliferating cells and the rate of their differentiation into hypertrophic cells, and calcification in the hypertrophic zone (Kobayashi et al., 2005).

Endochondral ossification and hypertrophy

There are just over two hundred bones in in the human body; all of them develop in one of two ways; either by endochondral ossification or intramembranous ossification (Lai and Mitchell, 2005). Endochondral ossification is the process by which the apical skeleton, vertebrae and base of the skull are formed. Intramembranous ossification is responsible for the formation of the facial bones the pelvis and the skull cap (Kelly and Jacobs, 2010). During both of these processes mesenchymal precursor cells begin the process by forming condensations, however, the rest of the two processes are very different (Kelly and Jacobs, 2010). During intramembranous ossification, the mesenchymal progenitors form vascularised matrix rich membranes, the mesenchymal cells within these membranes then differentiate directly into osteoblasts and convert the soft tissue membrane into bone (Buckwalter, 1996; Kelly and Jacobs, 2010). In order for bone to form through the process of endochondral ossification, first a primordial cartilage template called the anlagen must form; this provides the basis for the development of the resulting bone (Mau et al., 2007). This cartilage template is formed by mesenchymal precursor cells, and occurs through cell-cell and cell-matrix interactions that trigger condensation and the differentiation of mesenchymal precursors in to chondrocytes (Goldring et al., 2006). The expression of TGF-ß begins soon after condensation begins, and acts to define the outline of the condensation, and determine the final shape of the forming bone (Goldring et al., 2006). The newly formed chondrocytes secrete matrix molecules such as type II collagen and aggrecan. The expression of these chondrogenic markers is triggered by SOX9 which is up-regulated in the cells within the condensation (Mackie et al., 2011). At this stage of the condensates development, the centre of the condensate consists of proliferating chondrocytes, and the outer cells form a covering layer called the perichondrium (Lai, 2005). The first regions of calcified bone then begin to form the 'bone collar' around the diaphysis of the condensate. This ring of bone provides access for developing blood vessels which bring with them osteoblast precursors (Buckwalter, 1996; Mackie et al., 2011). The neovascularisation and development of osteoblasts which begin to remove the cartilage matrix leads to the development of the primary centre of ossification (Mau et al., 2007). At the epiphyses of the developing bones secondary centres of ossification begin to form. The movement of the primary and secondary centres towards each other leads to the creation of the cartilage growth plate, responsible for the elongation of the still developing long bones (Mackie et al., 2011).

The changes seen in cell morphology and organisation seen during the spread of ossification from the centres of ossification is very similar to those seen at the growth plate during bone lengthening. The resting zone chondrocytes show little organisation, as the cells become part of the proliferating zone they form columns and this structure is maintained throughout the process of ossification (Kobayashi et al., 2005).

In order for the cartilage anlagen to be converted into bone, the proliferating chondrocytes that formed the initial template have to go through a process called hypertrophy (Goldring et al., 2006). Hypertrophy is the final stage of the terminal differentiation of chondrocytes during endochondral ossification, and allows for the conversion of the cartilage template into bone (Fischer et al., 2010; Weiss et al., 2010). This stage of bone development, called hypertrophy, gets its name from the hypertrophy that the chondrocytes undergo as they differentiate. The volume of the cells can increase by up to twenty times; these chondrocytes begin to down-regulate the expression of chondrogenic markers such as type II collagen and aggrecan (Goldring et al., 2006; Mackie et al., 2011). As well as down-regulating chondrogenic markers there is an up-regulation in hypertrophic and osteogenic markers not normally seen in normal cartilage such as type I and type X collagen, Runx2, and ALP (Gauci et al., 2008). This expression

pattern is closer to that seen in osteoblasts than normal chondrocytes (Pelttari et al., 2006). The expression of Runx2 enhances the development of hypertrophic cells as it is a positive regulator for hypertrophic differentiation (Goldring et al., 2006). As the chondrocytes enter the later stages of hypertrophy, expression of the angiogenic factor VEGF (vascular endothelial growth factor) causes blood vessels to invade the hypertrophic zone (Goldring et al., 2006). The invasion of blood vessels brings with it osteoblast precursors (as during endochondral ossification), and leads to the calcification of the cartilaginous tissue. The expression of ALP by hypertrophic chondrocytes is key to the mineralisation of the hypertrophic zone, as the enzymatic activity of ALP clears the way for the process of calcification (Mackie et al., 2011). ALP encourages the formation of calcified tissue from the cartilaginous matrix by cleaving inorganic phosphates to form organic phosphates, and in doing so affects the balance of these two compounds in the developing bone (Orimo, 2010). The balance of these two forms of phosphate determines whether or not hydroxyapatite crystals can form (Orimo, 2010). By cleaving inorganic phosphate to organic phosphate ALP helps to provide conditions suitable to calcification (Orimo, 2010).

Following the invasion of blood vessels and osteogenic precursors, hypertrophic chondrocytes begin to undergo cell death (Mackie et al., 2011). As chondrocytes progress through hypertrophy cytomorphologic changes occur and two distinct populations begin to form, they are termed light and dark cells based on their differences when observed under a transmission electron microscope (Mackie et al., 2011). These two groups of chondrocytes undergo cell death in different ways (Mackie et al., 2011; Mueller and Tuan, 2008). Neither of the cells undergoes apoptosis; instead they have their own programs of cell death which vary from the classical form of apoptosis, referred to as 'chondroptosis' (Roach and Clarke, 2000). Both the dark and light cells demonstrate chromatin condensation as part of the cells death (Roach and Clarke, 2000). However, unlike the chromatin condensation seen at the periphery of the nucleus during the initial stages of apoptosis the chromatin of chondroptotic cells forms islands all through the nucleus (Roach and Clarke, 2000). Dark cells then begin to develop endoplasmic compartments and autophagic vacuoles that digest the cells contents, any remnants are then ejected into the cells lacunae (Roach et al., 2004). Light cells under-go a process in which the expansion of the rough endoplasmic leads to the formation of sacks around the organelles and other contents of the cell (Roach and Clarke, 2000). Lysosomes then release digestive enzymes that lead to the digestion of the cells contents within these cytoplasmic bodies (Roach and Clarke, 2000). The advantage of chondroptosis may be that autophagy removes the need for the inflammatory response required to remove the apoptotic bodies left behind by classical apoptosis (Roach et al., 2004). This response would be very difficult to mount in cartilage, and would therefore potentially lead to secondary necrosis, if the debris was not removed by phagocytosis (Roach et al., 2004).

Hypertrophy is clearly regulated by a number of factors, most notably the PTHrP/IHH axis, however there are other regulators e.g. triiodothyramine, a thyroid hormone which enhances hypertrophy (Mueller and Tuan, 2008; Mackie et al., 2011). The slowing of bone elongation and abnormalities of the growth plate are signs of hypothyroidism in humans (Mackie et al., 2011). There is also some evidence for the involvement of WNT activity in relation to hypertrophy (Kelly and Jacobs, 2010).The canonical WNT pathway regulates the expression of osteogenic genes by osteoblasts by regulating the intracellular levels of ß catenin, it has also been shown to play a role in promoting chondrocyte differentiation and hypertrophy (Kelly and Jacobs, 2010; Mackie et al., 2011).

Within bones and during the process of their development, there are two types of cartilage, transient cartilage, which forms the anlagen and is replaced by bone, and permanent cartilage that remains as hyaline articular cartilage at the distal ends of long bones and does not undergo ossification (Pelttari et al., 2008). Both of these types of cartilage form from mesenchymal precursors, but articular cartilage does not undergo the terminal differentiation seen in other chondrocytes. This is believed to be a result of a number of causes including the action of soluble factors on the chondrocytes (Fischer et al., 2010). Determining the causal factor or system that prevents articular chondrocytes from progressing to terminal differentiation would allow for the development of tissue engineering techniques that incorporate hypertrophy prevention. This would make a MSC cell therapy approach to cartilage repair more amenable to clinical use.

The major problem with using MSCs in cartilage therapy is that they undergo hypertrophy when they are cultured *in vitro* (Freyria and Mallein-Gerin, 2012). The reason that MSCs undergo hypertrophy is that once induced into the chondrogenic lineage it is the natural pathway for the chondrocytes, and chondrogenic MSCs to undergo hypertrophy and then for ossification of the tissue to occur along with chondrocyte cell death (Kim et al., 2008). This pathway seen in chondrogenically induced MSCs closely mimics the pathway taken by growth plate chondrocytes during bone development (Mueller and Tuan, 2008). The similarities between the two processes include the expression of certain genes for example: various isoforms of the FGF receptor, and markers of hypertrophy including MMP-13 (Mueller and Tuan, 2008). Hypertrophy does not develop in chondrogenically induced MSCs as a result of exposure to certain growth factors alone, although they can add to the effect, MSCs once induced into chondrogenesis will progress to hypertrophy in the absence of growth factors (Diekman et al., 2010).

One theory for the rapid progression of MSCs to

hypertrophy after chondrogenic induction *in vitro* is that all of the regulation and control systems seen within the processes of bone development are lost (Pelttari et al., 2006). As a result, rather than synchronously expressing type II collagen, aggrecan and other chondrogenic markers as a population in response to TGF-ß induced chondrogenesis, MSCs begin to express both chondrogenic and hypertrophic markers within days of TGF-ß exposure (Pelttari et al., 2006). Type X collagen was even detected before the full establishment of type II collagen production; this rapidly induces hypertrophy in the surrounding cells, and prevents MSCs from forming the stable pellets, capable of being transplanted into SCID mice that can be produced with articular chondrocytes (Pelttari et al., 2006). Should this simultaneous up-regulation of both chondrogenic and hypertrophic phenotypes be the cause of the hypertrophy seen in MSC cultures then there may be ways to tackle this differentiation, and enhance the cartilage production of MSCs.

Soluble factors are believed to play a major role in the preservation of the chondrogenic phenotype in articular cartilage (Fischer et al., 2010). When MSC pellets are cultured in medium conditioned by articular chondrocytes the expression of hypertrophic marker including type X collagen and IHH are lower than in MSC pellets cultured in unconditioned medium. This shows experimentally the importance of soluble factors (Fischer et al., 2010). This study also showed that when MSC pellets were cultured in a chondrogenic medium they only produced PTHrP for up to two weeks, after that, IHH signalling predominated (Fischer et al,. 2010). PTHrP has also been shown to be effective at down-regulating the expression of markers of hypertrophy in MSCs grown *in vitro* (Freyria and Mallein-Gerin, 2012). Methods for maintaining PTHrP expression, such as DNA transfection, or ways of inducing natural expression may help increase the chondrogenic stability of MSCs.

Other causes of the lack of stability in MSCs have been suggested. The natural environment for chondrocytes is hypoxic, at approximately 2-5% oxygen. Lowering the oxygen tension that MSCs induced into chondrogenesis are exposed leads to a down-regulation of markers of terminal differentiation.

Load may also play a role in maintaining MSCs in a chondrogenic state, computer modelling techniques such as finite element modelling (FEM), as well as studies applying loads to chondrogenic cells *in vitro* have shown that the application of low levels of compressive or sheer load to chondrogenic cells will result in the production of cartilage (Huang et al., 2004; Lee et al., 2011; Schätti et al., 2011).

There may also be other factors that are yet to be fully described or appreciated that are important to the successful maintenance of chondrogenesis in MSCs, e.g. small fragments produced by the digestion of type II collagen can cause an increase in matrix degradation and the differentiation of chondrocytes towards hypertrophy. This is related to data that has shown MMP-13 inhibitors can inhibit the hypertrophic differentiation of chondrocytes (Gauci et al., 2008).

An adjustment to the culture conditions, be it soluble factors in the culture medium that enhance chondrogenesis (e.g. IGF) or inhibit hypertrophy (e.g. PTHrP) or reducing the oxygen tension at which cells are cultured may prove to be very effective ways of reducing hypertrophy (Pelttari et al., 2008). Lowering the oxygen tension of culture conditions would better replicate the hypoxic environment of the healthy joint, and in doing so may help preserve the cells chondrogenic phenotype.

Scaffolds

As already mentioned the classical tissue engineering approach involves the implantation of cells capable of affecting a repair in to areas of damaged tissue. Although different implantation techniques are available, most of these use some form of scaffold to provide a particular physical or chemical environment for the cells. Scaffolds used in cell based techniques must have several features, they need to be biocompatible, allow cell adhesion and proliferation and preferably must be biodegradable as this allows the cells to produce extracellular matrix as the scaffold degrades, eventually leaving just repair tissue (Li et al., 2009). The choice of a particular scaffold from the huge range available is very important; as each type performs different functions, and affects cells differently e.g. certain hydrogels have been shown to enhance TGF-ß induced chondrogenesis in MSCs (Bosnakovski et al., 2006). The scaffold is important as it provides cells such as MSCs and chondrocytes with the 3D environment that they naturally inhabit *in vivo*, this 3D environment is crucial in a tissue engineering approach, as it allows for the correct differentiation, behaviour and phenotype maintenance of the cells being used to produce repair tissue.

Factors such as the porosity and connectivity of a scaffold can affect the fate decisions of cells within the scaffold (Panoskaltsis et al., 2005). Therefore, providing MSCs with a 3D environment that mimics their natural environment may encourage the cells proliferation and differentiation (Panoskaltsis et al., 2005).

Scaffolds are either derived from natural materials such as collagen or hyaluronan, or from synthetic materials such as poly-glycolic acid or poly-lactic acid (Redman et al., 2005). The natural scaffolds provide a more physiological environment e.g. type II collagen gels for chondrocytes, but the synthetic materials allow for greater flexibility e.g. *in situ* photopolymerisation (Klein et al., 2009). Scaffolds come not only in a suite of materials, but also in many different forms; most commonly hydrogels, fibrous sponges and hybrid constructs containing hydrogels set into sponges (Li et al., 2009).

One benefit of hydrogels is that they start off as

liquids, this allows for homogeneous cell distribution within the final implant as well as allowing for *in situ* cross linking (cross linking can be enzymatic, photo- or chemical) which provides superior defect filling (Klein et al., 2009). Hydrogels, particularly natural ones, also provide a physiologically suitable microenvironment for cellular activity (Ng et al., 2005). However, the mechanical strength of hydrogels is poor compared to articular cartilage, and in order to produce a stiff hydrogel e.g. from alginate, the concentration of polymer must be increased. This decreases the ability of fluid to flow through the construct, reducing nutrient delivery and waste disposal (Stoddart et al., 2009).

Sponge scaffolds, in contrast to hydrogels have good mechanical qualities and are able to cope with loading (Stoddart et al., 2009). Despite this however, the environment that sponges provide for cells is poorer than that provided by hydrogels in terms of attachment, cell fate and differentiation (Stoddart et al., 2009).

As a result of the different strengths of hydrogels and sponge scaffolds, they have been combined in to hybrid constructs that are capable of bearing load, as well as providing a suitable environment for the cells being transplanted into them (Stoddart et al., 2009). An example of this system was used by Li et al. (2009) in their loading experiments the group used a combination of polyurethane (PU) sponge, and fibrin hydrogel to culture and load chondrocytes. The PU confers load resistance to the scaffold whilst the fibrin helps to maintain the chondrocyte phenotype (Li et al., 2009).

Conclusion

There is a clear need for a clinical procedure that can reliably produce high quality hyaline like cartilage repair tissue within lesions of articular cartilage. Despite the range of current surgical techniques there is not currently a procedure among them that can consistently achieve this goal. The next step up from traditional surgical techniques is a cell based tissue engineering procedure. Currently the only form of this therapy available is autologous chondrocyte implantation. This procedure has been effective and has opened up the possibility of the use of cell based therapies in a clinical environment. However, this technique alone has been no more successful than current surgical techniques such as microfracture. ACI uses chondrocytes to produce repair tissue, an attractive alternative to chondrocytes are MSCs. These cells can be isolated in large numbers, particularly from bone marrow aspirates. As well as being isolated in large numbers these cells do not dedifferentiate in the same way that chondrocytes do when they are expanded in monolayer culture. Although they avoid some of the problems associated with chondrocytes, MSCs unlike chondrocytes undergo hypertrophy when they are induced into chondrogenesis using TGF-ß *in vitro*. Both physical and chemical stimuli have a dramatic effect on the fate determination of MSCs, and further research into these effects may provide a solution to this problem of hypertrophy. Of particular interest are PTHrP and IGF1 as these factors have been shown to prevent hypertrophy of MSCs *in vitro*. There may also be benefits to inducing chondrogenesis without TGF-ß as is current practise, but to instead use a form of loading to induce chondrogenesis through the endogenous production of TGF-ß.

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