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Review

Mesenchymal stem cells in connective tissue engineering and regenerative medicine: Applications in cartilage repair and osteoarthritis therapy

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Summary. Defects of load-bearing connective tissues such as articular cartilage, often result from trauma, degenerative or age-related disease. Osteoarthritis (OA) presents a major clinical challenge to clinicians due to the limited inherent repair capacity of articular cartilage. Articular cartilage defects are increasingly common among the elderly population causing pain, reduced joint function and significant disability among affected patients. The poor capacity for self-repair of chondral defects has resulted in the development of a large variety of treatment approaches including Autologous Chondrocyte Transplantation (ACT), microfracture and mosaicplasty methods. In ACT, a cartilage biopsy is taken from the patient and articular chondrocytes are isolated. The cells are then expanded after several passages *in vitro* and used to fill the cartilage defect. Since its introduction, ACT has become a widely applied surgical method with good to excellent clinical outcomes. More recently, classical ACT has been combined with tissue engineering and implantable scaffolds for improved results. However, there are still major problems associated with the ACT technique which relate mainly to chondrocyte de-differentiation during the expansion phase in monolayer culture and the poor integration of the implants into the surrounding cartilage tissue. Novel approaches using mesenchymal stem cells (MSCs) as an alternative cell source to patient derived chondrocytes are currently on trial. MSCs have shown significant potential for chondrogenesis in animal models. This review article discusses the potential of MSCs in tissue engineering and regenerative medicine and highlights their potential for cartilage repair and cell-based therapies for osteoarthritis and a range of related osteoarticular disorders.

Key words: Articular cartilage, Autologous chondrocyte transplantation, Mesenchymal stem cell, Mosaicplasty, Osteoarthritis, Regenerative medicine, Tissue engineering

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Introduction

Modern biomedical science has evolved at a staggering pace over the last century, especially in the last five decades. Significant advances have been made in the prevention, diagnosis and treatment of a range of infectious, neurodegenerative, cardiovascular, respiratory, renal, hepatic and diabetic diseases. For the past 160 years human life expectancy has increased by a quarter of a year every year (Oeppen and Vaupel, 2002). It is predicted that life expectancy will continue to increase by 2.5 years each decade, meaning that the western world's average life expectancy should reach and exceed 100 within the next 50 years (Oeppen and Vaupel, 2002). An important side-effect of such an increased lifespan in humans is the mounting burden of neoplastic, arthritic and rheumatic diseases. According to the World Health Organization (WHO), rheumatic or musculoskeletal conditions comprise over 150 diseases and syndromes, which are usually progressive and associated with pain. They can broadly be categorized as joint diseases, physical disability, spinal disorders, and conditions resulting from trauma. Musculoskeletal conditions are leading causes of morbidity and disability, giving rise to enormous healthcare expenditures and loss of work. Knowledge of the key determinants of

disability in musculoskeletal conditions is critical for reducing their burden on the world's growing population (Weigl et al., 2008). The number of rheumatoid arthritis (RA) and osteoarthritis (OA) patients steadily rises as the elderly population grows in Western Europe, North America and the rest of the developing world. RA, OA and back pain are important causes of disability-adjusted-life years in both the developed and developing world (Brooks, 2006). Back and knee pain are common in the community and are likely to increase with the ageing population (Brooks, 2006). Until recently OA was viewed as a "degenerative" or "wear-and-tear" disease and held little interest for most clinicians. In fact rheumatologists refused to have it classified as one of the conditions in their speciality until very recently. Veterinarians took an interest in it only because they had racehorses and racing greyhounds to take care of, whose economic value to their owners and the racing industries made it a worthwhile pursuit. Thus far human and veterinary clinical medicine has had little to offer, especially as OA was considered to be part of ageing and few distinctions were made in the clinical presentations.

It is now generally accepted that OA must be viewed not only as the final common pathway for ageing and injuries of the joint but also as an active joint disease. As the population of the world grows older and medical advances lengthen average life expectancy, osteoarthritis will become a larger public health problem - not because it is a manifestation of ageing but because it usually takes many years to reach clinical relevance. Osteoarthritis is already one of the ten most disabling diseases in developed countries.

The global pharmaceutical industries have been ignorant about the emergence of OA as a major musculoskeletal condition. They have in fact produced a limited portfolio of drugs for the treatment of arthritic conditions. However, the majority of the drugs they have offered for the treatment of arthritic and rheumatic diseases have been woefully inadequate as they only treat the symptoms of pain and inflammation. Although pharmaceutical companies have used a variety of methods including high throughput screening and combinatorial chemistry in their arthritis research programmes, thus far, they have failed to produce a single drug with the capacity to reverse the molecular changes that occur in OA. Consequently more effective function-modifying therapeutic strategies will need to be introduced for the clinical treatment of arthritic diseases such as OA. Connective tissue injuries are often very painful and connected with loss of biomechanical function, leading to restricted ability of patients to carry on with their daily routines.

Bone and cartilage defects are common features of joint diseases, such as rheumatoid arthritis and OA (Noel et al., 2002). They have a significant social and economic impact on the aging population. Despite progress in orthopaedic surgery, bone and cartilage repair is a major challenge as large defects will not spontaneously heal (Noel et al., 2002). Regenerative medicine is an emerging field that seeks to repair or replace injured tissues and organs through natural or bioengineered means. Recent research on stromal mesenchymal stem cells (MSCs) has provided a new and exciting opportunity for bone and cartilage tissue engineering. Thus far, MSCs have been isolated from bone marrow, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and deciduous teeth (Barry and Murphy, 2004; Sonoyama et al., 2006). MSCs possess the capacity to differentiate into cells of connective tissue lineages, including bone (Noel et al., 2002; Arinzeh, 2005; Hong et al., 2006), fat (Barry and Murphy, 2004; Helder et al., 2007), cartilage (Noel et al., 2002; Barry and Murphy, 2004; Caplan, 2007), intervertebral disc (Trubiani et al., 2005, 2006; Richardson et al., 2007), ligament (Trubiani et al., 2005, 2006; Sonoyama et al., 2006) and muscle (Ringe et al., 2002; Barry and Murphy, 2004). A great deal has been learnt about the isolation, cultivation and characterization of MSCs in recent years. A huge amount of research effort is focussed on their differentiation. MSCs have generated a great deal of public, scientific and media interest because of their potential use in regenerative medicine and tissue engineering. This review will focus on autologous chondrocyte transplantation (ACT) and selected tissue engineering methodologies that employ mesenchymal stem cells. The strengths and limitations of these approaches for articular cartilage regeneration and connective tissue repair will also be critically evaluated.

Osteoarthritis (OA)

Definition of OA

The word 'arthritis' literally means inflammation of synovial joints, and refers to a group of more than 200 diseases that affect the joints. Osteoarthritis (OA) is the most common type of arthritis. OA is the syndrome of joint pain and dysfunction caused by joint degeneration and affects more people than any other joint disease (Buckwalter and Martin, 2006). OA is primarily characterized by degeneration of articular cartilage (Roach et al., 2007), intra-articular inflammation with synovitis, and changes in peri-articular and subchondral bone (Goldring and Goldring, 2007). OA, also known as degenerative joint disease (DJD), may occur following traumatic injury to the joint, subsequent to an infection of the joint or simply as a result of ageing and the wear and tear associated with the stresses of daily life. Cartilage acts as a slippery cushion absorbing mechanical loads, thereby facilitating low friction movement of joints and allowing the ends of long bones to glide over one another. The breakdown of articular cartilage results in the loss of joint lubrication, causing the bones to rub against each other and form bone spurs or osteophytes. In some cases fragments of cartilage can break off and float inside the joint space causing obstruction, inflammation and further structural damage.

The main symptoms of osteoarthritis are pain, stiffness and swelling of the joints. The joint may have restricted movement, and there may be tenderness or deformity. The joint may also crack or creak a phenomenon that is often described as crepitation (Stegenga et al., 1991; Broussard, 2005). When the joint becomes severely damaged, it may become misshapen, with numerous osteophytes, leading to further instability. This puts stress on the ligaments and tissues surrounding the joints, and can lead to deformity.

Demographics of OA

OA is rare in people under 40 but becomes more common with age - most people over 65 years of age show some radiographic evidence of OA in at least one or more joints. OA is the most frequent cause of physical disability among older adults globally. More than 8 million people in the UK and over 20 million Americans are estimated to have OA. It is anticipated that by the year 2030, 20% of adults will have developed OA in Western Europe and North America. OA is not only a common problem among the elderly population, but also it is becoming more widespread among younger people. In the United States, RA and OA combined affect as many as 46 million people. This amounted to a healthcare cost of over $128 billion in 2003. This huge financial burden emphasizes the acute need for new and more effective treatments for articular cartilage defects. Furthermore, there are currently no disease modifying drugs or treatments for OA. Existing pharmaceuticals include steroids and non-steroidal anti inflammatory drugs which are unsatisfactory and only treat the symptoms of OA by reducing pain and inflammation. Therefore, OA represents a major opportunity for research and development. Any new information gained about new treatments developed for treating OA in human patients will also have benefits to companion animals such as horses and dogs which also suffer from OA.

Established treatments for OA

Sensible treatments for OA include preventive measures, i.e. removal or treatment of the inciting cause; restoring joint stability; treating underlying metabolic or endocrine diseases that are known to exacerbate OA. Another major contributor to OA is obesity (Abramson et al., 2006; Hunter, 2008). Therefore, weight control and exercise regimes are recommended for overweight patients with OA. A number of surgical methods and procedures have been implemented to restore synovial joint function. These range from minimally invasive procedures such as arthroscopic abrasion (debridement) and shaving of small cartilage defects, to more extended surgical procedures such as microfracture of the subchondoral bone and mosaicplasty. Surgery for OA, which may involve joint replacement, is generally unsatisfactory. Hip replacements are increasingly common and give OA patients a new lease of life, with improved mobility and significant pain relief. Hip replacements are usually effective for at least 10 years - after this, they may need to be replaced. Replacing the knee is a much more complicated procedure, since the knee joint is more complex than the hip joint. Nevertheless, knee replacements can also bring OA patients significant improvements in their quality-of-life. Alternative and complementary therapies for OA are controversial. Nutritional and nutraceutical therapies have been proposed for OA. However, the use of nutraceuticals is highly controversial and is debated elsewhere (Hauselmann, 2001; Ringe et al., 2002; Barry and Murphy, 2004; Goggs et al., 2005; Henrotin et al., 2005; Trumble, 2005; McAlindon, 2006; Clark, 2007; Frech and Clegg, 2007). Unfortunately, since joints have a very poor capacity for healing and repair, cartilage regeneration is generally minimal due to the limited repair capacity of the cartilage tissue. The resulting repair tissue often lacks the mechanical properties and physical durability of the original articular cartilage. This results in further degeneration of the cartilage and continued decline in joint function.

In recent years, a range of methods have been developed for the repair of articular cartilage lesions. These include osteochondral transplantation, micro-fracture and autologous chondrocyte transplantation (ACT), with or without the assistance of a scaffold matrix to deliver the cells. A feature of all of these techniques is that their use is limited to the repair of focal lesions and patients with OA are mostly excluded from treatment. OA cartilage lesions are generally large and unconfined and so do not provide an appropriate environment for chondrocytes to be retained long enough to synthesize an extracellular matrix. Therefore, successful repair of OA cartilage lesions is only likely to be achieved when 3-dimensional cartilage implants can be generated that have enough extracellular matrix for fixation within the joint.

Novel and innovative treatments for OA

ACT has been in clinical use for a decade but has several major drawbacks. Challenges in treating cartilage defects with ACT include paucity of the cell source; damage caused to native tissues by cell harvest; inability to restore the original cartilage structure (40% of ACTs show evidence of chondrocytes hypertrophy); lack of adhesion between new repair cartilage and the original tissue. In general, most tissue engineered scaffolds do not possess the appropriate biomechanical properties (i.e. they are not sufficiently load-bearing). The advent of mesenchymal stem cells (MSCs) and tissue engineering has provided osteoarticular pathologists, cell biologists and clinicians a new and exciting medium for experimentation. It is often asked: 'Can tissue engineering and MSCs be used to treat OA?' The optimistic answer is of course, 'yes'. However, the realistic answer should be 'possibly' or 'hopefully'.

Stem cells and tissue engineering for large bone defects

Another expanding area of research and clinical development in connective tissue medicine and biology is the healing of large bone defects. Large bone defects, mainly those resulting from trauma, loss of large bone areas after cancer surgery, or bone loss through metabolic disorders connected with weakening of the whole bone structure, require special treatments. Clinical approaches include allografting and have established themselves in routine clinical medicine. However, the incidence of donor site morbidity, risk of infection from allografts or sheer implant size have meant that reconstructive medicine has reached bottle-neck limitations. These requirements have guided the development of new strategies, most of these evolving around bio-compatible and bio-degradable scaffold construction and seeding of scaffolds *in vitro* with appropriate cells such as primary osteocytes or mesenchymal stem cells (Krampera et al., 2006).

Potential of stem cells in treating tendinopathies

Tendon injuries are a major problem in clinical medicine. Tendon injuries are often the consequence of recurrent micro- or macro-traumata, continued mechanical over straining or the side effect of other medical therapies such as antibiotic treatment with gyrase inhibitors such fluoroquinolones (Shakibaei et al., 2000, 2001a; Shakibaei and Stahlmann, 2001, 2003; Sendzik et al., 2005; Mehlhorn and Brown, 2007) . Strategies of tendon repair evolve mainly around allografting of tendons. In recent times, new approaches using bio-compatible and bio-degradable scaffolds and *in vitro* tissue engineering of tendon from tenocytes or MSCs have been introduced and evaluated (Krampera et al., 2006).

Challenges in connective tissue healing (bone, tendon, cartilage)

There are a variety of problems associated with the healing of connective tissues such as bone, tendon and cartilage. Bone, compared to tendon and cartilage, heals relatively quickly, through regenerating and remodelling itself. The major advantage of bone tissue is its good vascularisation, not only making it possible for new cells to reach the site of a defect but also to remove and dispose of apoptotic and necrotic tissue. However, large bone defects resulting through trauma, tumors or metabolic and degenerative diseases have a limited capacity for self repair. This highlights the acute need for readily available, implantable bone grafts (Beris et al., 2005; Kajiwara et al., 2005).

Autogenous and/or allogenic osteochondral grafts have been used for several decades to fill osteochondral defects. A wealth of clinical experience has thus far been gathered. Patients with large defects such as large osteochondoral defects after tumor resection, osteonecrosis, extensive trauma or broad focal OA have especially benefited from these grafts (Meyers et al., 1983; Czitrom et al., 1986; Marco et al., 1993). However, autogenous grafts are frequently connected with donor site morbidity (Hangody and Fules, 2003). Furthermore, allografts always increase the risk of infections and they must be immunocompatible with the patient to avoid implant rejection and peripheral bone necrosis. These problems have led to new research to provide bone substitutes produced *in vitro* through tissue engineering. Today, bone tissue engineering is a dynamic and rapidly expanding field. The main focus is on scaffolds which provide an artificial extracellular matrix for the colonization of appropriate cells such as primary osteoblasts and various combinations of growth factors.
Tendon and cartilage exhibit bradytroph properties *in vivo* and possess very limited regeneration capacities (Schulze-Tanzil et al., 2004a). Tendons represent an essential part of the musculoskeletal system linking the dynamic (muscles) to the static (bone) components. To be able to meet the demand of the mechanical environment, tendons must have a highly specialized extracellular matrix (ECM), consisting of parallel aligned fibres capable of withstanding tensile loads. Thus, tendon consists of large quantities of ECM and few cells (tenocytes) which produce this highly specialised matrix. More than 95% of tendon ECM is made up of collagen type I, but other collagens (type III and V) as well as proteoglycans, elastin, fibronectin are also present (Kannus, 2000; Canty and Kadler, 2002; Schulze-Tanzil et al., 2004a). Its marginal vascularisation and the low mitotic activity of the tenocytes are major factors and contributors to the poor repair and regeneration potential of tendons (Ahmed et al., 1998).

Cartilage is another connective tissue that owes its special characteristics to the dense ECM produced by chondrocytes. About 40 to 50% of cartilage ECM consists of collagens (of these, approximately 90% is collagen type II) and about 20 to 25% of different proteoglycans (aggrecan, decorin, biglycan and fibromodulin). A particular feature of its cells, the chondrocytes (which make up less than 5% of the tissue's total 3-dimensional volume) is that they do not have any direct cell-to-cell contact with each other. Thus, each cell may be regarded as a functional unit responsible for maintaining the ECM in its immediate surrounding through balanced and tightly regulated anabolic and catabolic activities. Cartilage is avascular, aneural and alymphatic (Bora and Miller, 1987). This unique composition further explains the limited repair capacity of articular cartilage and why most repair tissues fail due to the dominance of a fibroblast-like cell type which produces an ECM without the necessary biomechanical properties of hyaline cartilage (Vachon et al., 1986; Cancedda et al., 2003).

During embryogenesis, cartilage is formed from the condensation of mesenchymal stem cells (MSCs). Mesenchymal cell aggregates, termed blastema *in vivo*, precede cartilage differentiation *in vivo* and also in high-density cell cultures (Aulthouse and Solursh, 1987;

Solursh, 1989). This process is characterized by the production of cartilage matrix specific proteoglycans and a switch from collagen type I to collagen type II synthesis (Cancedda et al., 1995). In articular cartilage chondrocytes stay at a mature state. However, in adolescents during endochondral bone development, chondrocytes become hypertrophic, produce alkaline phosphatase and collagen X and are eventually reabsorbed while new bone is formed in the growth plate.

Clinical methods for cartilage repair: Autologous Cartilage Transplantation (ACT)

Throughout life our bodies are constantly exposed to external and internal stresses. In order to survive and maintain optimal health, it is essential for the body to be able to repair and regenerate damaged tissues. This is normally achieved by replacement of dead, dying and damaged cells with progenitor cells that have the capacity to differentiate into the specialised cell type being replaced. For example, in tissues such as the intestine or the skin, regeneration is accomplished via proliferation and differentiation of unspecialised multipotent resident cells, the stem cells (Tallheden et al., 2006).

As discussed earlier, articular cartilage is a poorly vascularised tissue and when it is damaged chondroprogenitor cell access to the damaged site will be very limited. Consequently, a new method termed Autologous Cartilage Transplantation (ACT) was introduced by a Swedish group following the general principles of tissue repair (Brittberg et al., 1994). ACT was designed as a novel clinical treatment for articular cartilage repair to solve the problem of progressive degeneration in OA joints. The idea was to fill up the cartilage defect with autologous chondrocytes (i.e. derived from the same patient), combining surgical treatment with *in vitro* methods. The chondrocytes would be placed into the defect and through this transplantation cell condensation would be triggered, mimicking the condensation phase in early embryonic development. This condensation phase would give the chondrocytes a new stimulus for matrix production and hence lead to restitution of hyaline cartilage tissue in the cartilage defect. Today, many modifications of the technique exist and are used in the clinic. The basic techniques for the clinical application of chondrocyte implantation has been re-evaluated by Brittberg who has also provided an update on the clinical results (Brittberg, 2008).

The ACT method is briefly summarized in this section. A biopsy (ca. 150-300mg cartilage tissue) is surgically taken from a non weight bearing area of the affected joint, for example from the supromedial edge of the femoral condyle in the knee, and transferred to a special sterile, nutrient solution for transport. In the cell culture laboratory, chondrocytes are then isolated from the cartilage tissue through enzymatic digestion with collagenase and pronase. The chondrocytes are then seeded *in vitro* in monolayer culture and expanded. The goal of the *in vitro* expansion is to obtain a sufficient number of cells for re-implantation to be able to fill up the cartilage defect. In a second surgical procedure, the *in vitro* expanded chondrocytes are then injected into the defect. To secure the chondrocytes remaining at the implanted side and to prevent the mass from floating away, a periosteal flap is further sewed over the defect (Brittberg et al., 1994). It is well documented that the periosteal flap alone can have chondrogenic capacities, and induce cartilage regeneration. However the precise role of the periosteum still remains to be elucidated.

Results of long-term studies

In the first animal experiments performed on rabbits, the ACT technique was performed on chondral defects that had not penetrated the subchondral bone. These results were very encouraging; the rabbits showed new cartilage formation in 82% of the defect area (Grande et al., 1989). In further studies, chondral defects of the patella in rabbits were either treated with chondrocytes or left empty with only the periosteal flap covering the defect (Brittberg et al., 1996), or scaffolds were used with chondrocytes seeded into an agarose gel and then transplanted (Rahfoth et al., 1998). In both cases, the one year outcome showed significantly higher hyaline cartilage production in treatments with added chondrocytes compared to control treatments without cells (between 47 to 87%). To evaluate whether the implanted chondrocytes stayed at the implantation site or whether tissue repair was performed by other cells, chondrocytes were membrane labelled with a fluorescent dye to track them after implantation *in vivo*. A six week outcome in a goat model showed that cells persisted in the defect site (Dell'Accio et al., 2003). In contrast to the rabbit studies, a canine model showed no significant difference between the ACT treated areas and the controls (Breinan et al., 1997). However, in a canine study, a scaffold seeded with chondrocytes was used which showed significantly higher values of defect regeneration (42% of defect area filled with hyaline cartilage) (Lee et al., 2003).

ACT has been in clinical use in human patients since 1987 and has been performed on over 12,000 patients worldwide (Peterson et al., 2002). ACT has significantly reduced pain in patients - even the production of durable cartilage-like tissue has been observed (Peterson et al., 2002). In human patients, results after three to nine years are very encouraging, although repair of the defect is not uniform in all areas of the joint (Brittberg et al., 1994; Peterson et al., 2000). Although clinical results are encouraging and overall the patients are satisfied, it must be borne in mind that there is still a lack of comparative, blinded, long term group studies in human subjects.

Problems with ACT

Despite the encouraging clinical results there are still limitations to the use of ACT. These are mainly related

to: a) the complexity of the surgical procedure, b) the biological response of the periosteal flap, and c) the de-differentiation and consequent capacity loss associated with *in vitro* expansion of isolated chondrocytes (Schulze-Tanzil et al., 2002, 2004b; Brittberg et al., 2003; Dell'Accio et al., 2003). Most clinical complications associated with ACT in fact are connected to the periosteal flap. These include periosteal flap detachment, delamination and late periosteal hypertrophy (Brittberg, 1999).

One of the main hurdles to successful cartilage repair is chondrocyte dedifferentiation during the monolayer expansion phase. Within articular cartilage, chondrocytes have a distinct round to oval morphology; cells remain embedded close to their surrounding ECM. The tight interaction between chondrocytes and the ECM represents a major factor in the maintenance of chondrocyte function, vitality and its unique biosynthetic programme (Shakibaei et al., 1993, 1997; Shakibaei and De Souza, 1997; Shakibaei and Merker, 1999). Damaged ECM or its complete absence will result in a major shift in chondrocyte gene expression. Instead of producing cartilage specific proteoglycans and collagen type II, chondrocytes switch to making non-specific proteoglycans and collagen type I (von der Mark et al., 1977; von der Mark, 1980; Marlovits et al., 2004). These matrix components lack the biomechanical properties and the resilience of articular cartilage. The monolayer culture conditions *in vitro*, where chondrocytes are forced to give up their round shape in order to adhere to the plastic in order to survive, are a key component of chondrocyte de-differentiation. This becomes phenotypically evident after the cells adhere to tissue culture plastic and continue de-differentiating with prolonged culture. These de-differentiated cells are no longer capable of re-differentiation when re-implanted in the cartilage defect. Re-differentiation in 3-dimensional surroundings of the cultured chondrocytes can be achieved up to the fourth passage in monolayer (Schulze-Tanzil et al., 2002). Furthermore, growth factors are known to be involved in the re-differentiation of chondrocytes. It has been shown that the insulin like growth factor I (IGF-I) as well as transforming growth factor beta (TGF-ß) influence and modulate the collagen network in cartilage and can prolong the re-differentiation capacity of monolayer expanded chondrocytes (Hunziker, 2001; Barbero et al., 2003; Jenniskens et al., 2006). Indeed, it has been shown that treatment of monolayer cultured chondrocytes with IGF-I will prolong their re-differentiation potential. After a protracted monolayer expansion phase, IGF-I treated chondrocytes are still able to produce specific cartilage matrix components in 3-dimensional conditions in comparison to chondrocytes not exposed to IGF-I (Shakibaei et al., 2006).

New perspectives on classical ACT

New approaches *in vivo* and *in vitro* to modify the classical ACT method have been examined. These so called second and third generation ACTs include various biomaterials to replace the peristoeal flap and a large variety of scaffold materials as chondrocyte carriers to the defect site (Marlovits et al., 2006). Furthermore, *in vitro* studies have been implemented to study the molecular mechanisms and signal transduction pathways involved in chondrogenesis to precisely influence de-differentiation of chondrocytes.

Second generation ACT uses a bi-layer collagen type I/type III membrane (such as Chondro-Gide™) to be sutured over the defect instead of the periosteal flap. The cell suspension is then injected underneath. Using a collagen membrane brings the added advantage of reducing the time needed for surgical procedures, since the preparation of the periosteal flap is no longer necessary. Furthermore, using the collagen membrane reduces the complications at the implant site connected with the periosteal flap usage (i.e. periosteal hypertrophy).

The so-called third generation ACT goes one step further by attempting tissue engineering cartilage *in vitro* through culturing chondrocytes on scaffolds. In general, tissue engineering can be defined as the art of reconstructing mammalian tissue, both structurally and functionally (Hollander et al., 2006). This reconstruction process can be performed entirely *in vitro*, with whole new mature tissue being transplanted or partially *in vitro* and the not fully matured tissue construct matured further after transplantation *in vivo*. Naturally success in tissue engineering of any connective tissue would make tissue transplantation and grafting redundant.

In the case of cartilage tissue engineering, after expansion in monolayer culture, chondrocytes are seeded onto a 3-dimensional scaffold before transplantation into the defect. The properties of these scaffolds follow basic principles; they must be biocompatible, structurally and mechanically stable and must support the loading of an appropriate cell source to allow successful infiltration and attachment to the host tissue (Tuli et al., 2003). The composition of these scaffold materials varies greatly and a large variety have been tested *in vitro* and *in vivo* (Hunziker, 2002). There are a wide variety of materials used such as polylactide-co-glycolide (PLG) based (Mercier et al., 2005), hyaluronan-based (Solchaga et al., 2005b) or atelocollagen based (Ochi et al., 2002) scaffolds. Studies on animals (Wakitani et al., 1998; Solchaga et al., 2005b) and human patients (Ochi et al., 2002) have been performed. Results were positive, but some human patients showed signs of hypertrophy and partial ossification of the implants (Ochi et al., 2002).

Cartilage tissue engineering has been attempted with scaffold free techniques (Marlovits et al., 2003; Kelm and Fussenegger, 2004) and applied in animal models (Mainil-Varlet et al., 2001). For example, autologous cartilage implants fixed with titan-suture-anchors have been tested in horses where histological analysis of one euthanized horse showed repair of a defect with a tissue

similar to native cartilage after a period of 24 months. Nevertheless, other problems with ACT still remain, such as poor integration of repair tissue into the surrounding cartilage. This limitation has been recognized by several authors (Ahsan et al., 1999; Hunziker, 2001, 2002). Histological staining reveals a clear margin between the implant and the native tissue and often there is apoptosis and necrosis at the interface between the two. Although various measures, such as collagen-cross linkers and biological glues have been used to enhance tissue integration after implantation (Grande and Pitman, 1988; Jurgensen et al., 1997; Ahsan et al., 1999) they have been relatively unsuccessful. Furthermore, the required size of the tissue engineered constructs is often overlooked ((Hunziker, 2002). This is particularly important when comparing the size of experimental animals (such as rabbit and goat) and their artificially induced defects with the size of human chondral lesions. Whether large enough tissue engineering constructs are able to be created for human patients is an important question that highlights many problems. These problems focus on the properties of large implants to withstand mechanical loading and stress, survival of the cells in the tissue engineered implant and thus survival of the implant tissue.

The above mentioned problems are connected mainly with physical and clinical procedures. However, new strategies involving ACT improvement have to center around enhancing and prolonging the chondrogenic potential of the chondrocytes during their *in vitro* expansion phase. This is of vital importance since the core component of hyaline cartilage regeneration is dependent on the potential of the chondrocytes to re-differentiate and produce adequate hyaline cartilage matrix. As stated above, the chondrocytes from the biopsy have to be expanded in monolayer to yield enough chondrocytes to fill up the cartilage defect. However, this expansion phase leads to a loss of their chondrogenic phenotype and re-differentiation potential and thus results in the chondrocytes being incapable of cartilage production after *in vivo* implantation (Schulze-Tanzil et al., 2002, 2004b). To achieve success in tissue engineering it is important to gain a better understanding of the biochemical and molecular signaling pathways involved in chondrogenesis. This knowledge would enable scientists to specifically stimulate these pathways and systematically induce the cells to re-differentiate into cartilage matrix producing chondrocytes.

Several *in vitro* studies have been performed to better understand chondrocyte physiology and the molecular signaling pathways involved in cartilage differentiation. A major signaling pathway involved in activation of the chondrogenic differentiation of chondrocytes is the MAPkinase pathway (Shakibaei et al., 2001b; Schulze-Tanzil et al., 2004b). This pathway stimulates the major chondrogenic transcription factor Sox9. The MAPkinase pathway can be stimulated through a variety of factors. Growth factors, such a IGF-I and TGF-ß, have been proven to interact with their cell surface receptors and stimulate the adaptor protein Shc (Shakibaei et al., 2006). Stimulation of Shc leads to subsequent stimulation of ERK1/2 and further kinases of the MAPK pathway. *In vitro* stimulation of monolayer chondrocytes with IGF-I prolongs their potential to re-differentiate and produce cartilage specific matrix components such as collagen type II and cartilage specific proteoglycans (Shakibaei et al., 2006). This approach could become a valuable tool for future applied ACT strategies, since it provides adequate numbers of chondrocytes that are still able to re-differentiate in vivo and thus produce adequate hyaline cartilage matrix. This would provide a new, sound basis for chondrocyte-based cartilage tissue repair.

Other methods for cartilage repair

In the following sections we define and discuss microfracture and mosaicplasty before reviewing and justifying the use of mesenchymal stem cells for cartilage repair.

Microfracture

There are a variety of other techniques used to attempt clinical cartilage regeneration (Hunziker, 2002). Two of these; the microfracture method and the mosaicplasty method, will be briefly discussed here. The microfracture technique is an intervention which does not use biological agents, such as chondrocyte implants. During the surgical procedure, the surgeon drills small holes into the surface of the cartilage over the defect area. The holes are 3-4mm apart and 4mm deep. The rationale behind this is that the drilling of the holes stimulates a spontaneous repair reaction. It is notable that the microfracture technique has been mainly applied in young individuals and athletes, with whom it yields relatively good results in regard to regaining of joint function and pain relief (ca. 75%) (Sledge, 2001). However, no studies on OA patients exist. Thus, it remains questionable whether microfracture can achieve such good results in OA patients where the regenerative potential of cartilage is already diminished.

Mosaicplasty

Mosaicplasty or Autogenous OsteoChondral Grafting (AOCG) is a breakthrough technique which is the result of more than a decade of animal and clinical research and refinements in surgical technique and instrumentation. It has been in clinical use since 1992 and has been shown to be an efficacious, reproducible, and cost-effective means of restoring chondral defects. This procedure is indicated for the treatment of focal, full thickness, cartilage lesions of up to 9 cm in the knee and ankle. Mosaicplasty basically involves the implantation of whole osteochondral tissue into the cartilage defect. The idea of implanting autologous or

allogenic osteochondral grafts to repair cartilage defects dates back to the beginning of the last century. This technique has been clinically tried, tested and refined. Thus far there is a considerable amount of clinical data available on this technique (Hangody et al., 2001a,b). In mosaicplasty round 'plugs' of cartilage and underlying bone are excised from healthy non-weight-bearing areas and moved to damaged areas. The plugs are each a few millimeters in diameter, and when multiple plugs are moved into a damaged area the result is a mosaic appearance-the multiple small plugs of cartilage look like mosaic tiles. The first step is to prepare the area of damaged cartilage. A coring tool is used to make a perfectly round hole in the bone in the area of damage. This hole is sized to fit the plug. The second step is to 'harvest' the plug of normal cartilage. The plug is taken with the underlying bone to fit into the hole that was prepared in the area of damage. The plug is just slightly larger than the hole so it will fit snugly into position. The final step is to implant the harvested plugs into the hole that was created in the damaged area. Over time, the hope is that the implanted bone and cartilage will incorporate and integrate into its new environment.

The results of mosaicplasty appear promising with 60 to 90% of the patients reporting pain relief and restored joint function. However, no blinded clinical trials been done and therefore results must be regarded with caution. Animal studies have been performed on both small experimental animals such as rabbits and on large animals such as the horse. The successful treatment of an equine subchondral cystic lesion with mosaicplasty has been reported (Bodo et al., 2000). Although a study with horses reported resistant and consistent integration of the osteogenic part of the graft for a long period of time, the cartilage part of the graft was resorbed after 6 months. Studies with the sheep have given similar results, showing not only resorption of the chondral part of the graft, but also degeneration of the adjoining cartilage tissue (Wohl et al., 1998; Bodo et al., 2000). The poor integration of the implants into the defects is connected to the translocation of a graft from a non weight bearing area to a weight bearing area, leading to physiological stress through mechanical overloading of the cartilage. Furthermore, the tissue can be damaged when the implants are hammered into the defect by the transplanting surgeons (Quinn et al., 1998a,b). Therefore, the results of the mosaicplasty method appear ambiguous and must be regarded with caution.

Stem cells for connective tissue regenerating medicine and cartilage repair

There are several limiting factors when using autologous chondrocytes. Each biopsy is an additional trauma to cartilage that is already damaged and injured in the joint. Furthermore, suitable donor sites for hyaline cartilage are rare in damaged joints, and as yet, other cartilage has not been fully assessed for its ability to repair articular cartilage (hyaline nasal or rib cartilage) (Naumann et al., 2002). In addition, the number of cells obtained via biopsy is relatively small and therefore the cells have to be expanded for several passages. Chondrocytes grown in monolayer de-differentiate with passaging and subsequently lose their chondrogenic phenotype and their re-differentiation potential. This loss of cartilage specific matrix components is indicated mainly through a switch in the cells from producing collagen type II to collagen type I (von der Mark et al., 1977; Darling and Athanasiou, 2005). It has been shown that re-differentiation is possible with the help of specific growth factors such as TGF-ß (Denker et al., 1999; Jakob et al., 2001; Barbero et al., 2003) as well as without growth factors (Anderer and Libera, 2002). However to avoid further distress for the traumatized joint and initially obtain high numbers of cells, an alternative source of cells with chondroprogenitor potential would be useful. One such possibility for the construction of neo-cartilaginous tissue is the use of mesenchymal stem cells (Caplan and Goldberg, 1999).

What are stem cells?

Tissue engineering has rapidly evolved as a new and exciting field of basic research, investigating how to repair and regenerate tissues by mimicking *in vitro* key features of developmental pathways in tissue formation using a combination of growth factors and biomaterials (Caplan and Goldberg, 1999). In the last decade a new understanding of embryonic development has led to the therapeutic approach of repairing skeletal tissue through tissue engineering and the reconstruction of pieces of cartilage and bone for subsequent implantation has opened up whole new regenerative medical strategies (Caplan, 1991, 2000, 2007; Cancedda et al., 2003; Short et al., 2003).

Stem cells have the remarkable potential to develop into many different cell types in the body. The various different applications of cultured stem cells have been summarized in Figure 1. In addition to serving as a sort of repair system for the body, they can theoretically divide without limit to replenish other cells as long as the person or animal is still alive. Stem cells have two fundamental characteristics that distinguish them from other types of cells. First, they are unspecialized cells that renew themselves for long periods through cell division. The second is that under certain conditions they can be induced to become specialized cells with unique biological functions (Fig. 1).

An un-differentiated progenitor cell, ideally present everywhere in the body and yet capable of differentiating into various mesenchymal, epidermal and endodermal specialized tissue/organ cells is the ideal stock material from which an *in vitro* tissue engineered tissue replacement could be made (Fig. 2). Stem cells are a promising cell source that fulfill these requirements for tissue engineering. In general, a stem cell is described as an unspecialized progenitor cell that resides in niches in various organs and tissues from where it can be recruited

to replenish specific tissue cells when they die (Fuchs et al., 2004; Caplan and Dennis, 2006). Stem cells can be found in the embryo, fetus and the adult individual. Stem cells are capable of differentiation, therefore using stem cells from the adult individual has two advantages: firstly, the ethical considerations which are associated with attaining stem cells from the embryo are redundant and secondly, autologous cells could be recovered for tissue engineering implant production thereby circumventing any possible immunomodulatory responses after allogenic graft implantation. However, in contrast to embryonic stem cells which are pluripotent, adult or fetal stem cells are multipotent and hence have reduced differentiation capacities (Fig. 2). In adult individuals, stem cells have been identified in various organs and tissues. The earliest identified stem cells belong to the hematopoietic lineage and are all derived from hemocytoblasts in the bone marrow. Hematopoietic

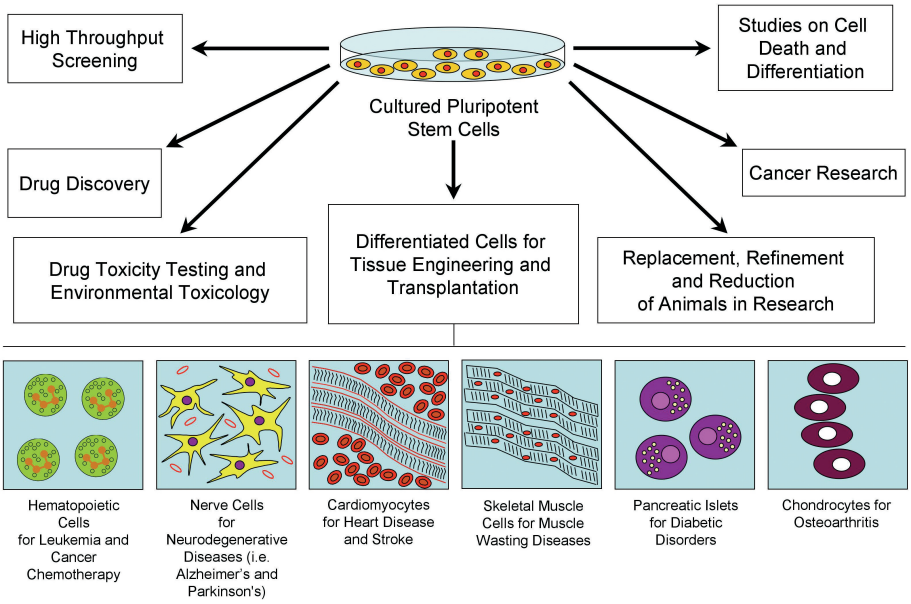

Fig. 1. Applications of cultured pluripotent stem cells. The various applications of stem cells are summarized in this schematic.

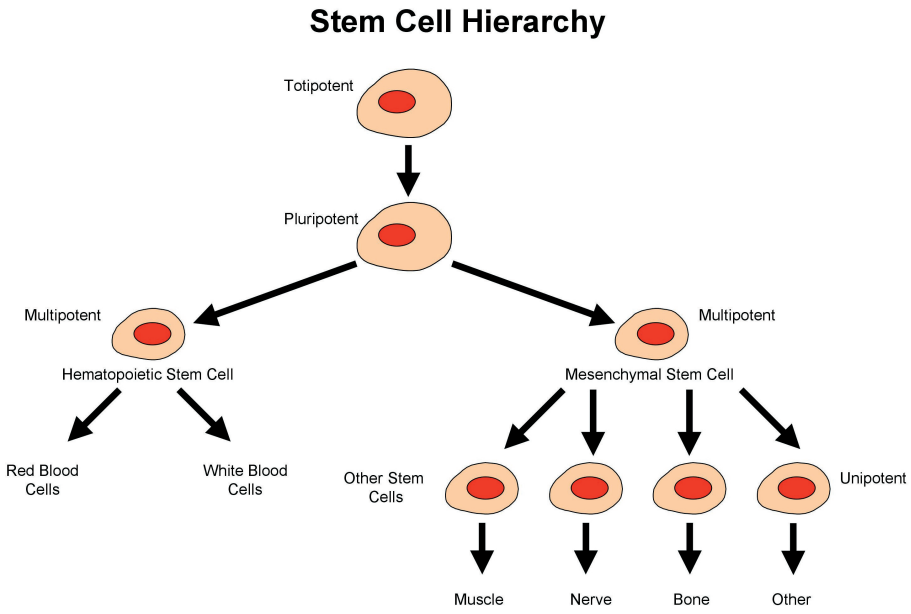

Fig 2. The hierarchy of stem cells. The potency of stem cells specifies the differentiation potential of stem cells. Totipotent stem cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent. These cells can differentiate into embryonic and extra-embryonic cell types. Pluripotent stem cells are the descendants of totipotent cells and can differentiate into cells derived from any of the three germ layers. Multipotent stem cells can produce only cells of a closely related family of cells (e.g. hematopoietic stem cells differentiate into red blood cells, white blood cells, platelets, etc. and other stem cells which include mesenchymal stem cells). Unipotent cells only have the capacity to produce one cell type, but have the property of self-renewal which distinguishes them from non-stem cells (e.g. muscle stem cells).

stem cells give rise to all the blood cell types including myeloid and lymphoid lineages (Fig. 3). The definition of hematopoietic stem cells has undergone considerable revision in the last two decades. The hematopoietic tissue contains cells with long term and short term regeneration capacities and committed multipotent, oligopotent and unipotent progenitors.

Today, adult stem cells have been isolated from a large variety of organs including peripheral blood, bone marrow, muscle, fat, pancreas, skin, the central nervous system and many other tissues (Till and McCulloch, 1980; Caplan, 1991; Williams et al., 1999; Zuk et al., 2001; Alexanian and Sieber-Blum, 2003; Bottai et al., 2003; Cancedda et al., 2003). Over the last two decades it was discovered that the bone marrow hosts not only hematopoietic stem cells but also contains a pluripotent mesenchymal fibroblastic progenitor cell type that can differentiate towards bone, muscle, fat, cartilage and other connective tissues *in vitro* (Caplan, 1991; Fortier et al., 1998; Conget and Minguell, 1999; Pittenger et al.,

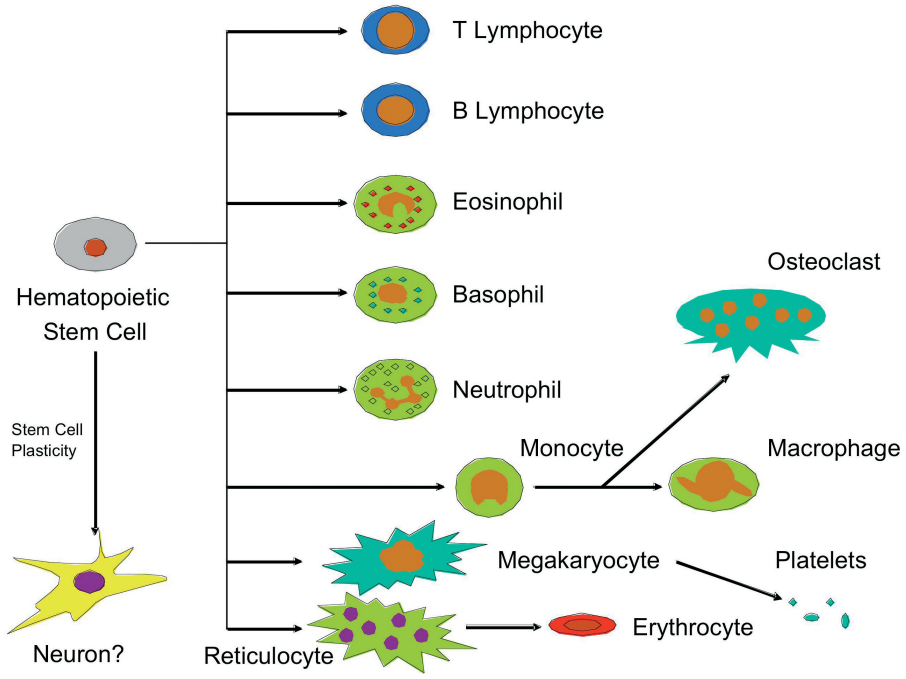

Fig. 3. Differentiation of hematopoietic stem cells. Hematopoietic stem cells are multipotent stem cells which have the capacity to give rise to all the blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells) and lymphoid lineages (T-cells, B-cells, NK-cells). They may also have the capacity to give rise to neuronal cells through a poorly understood process known as stem cell plasticity.

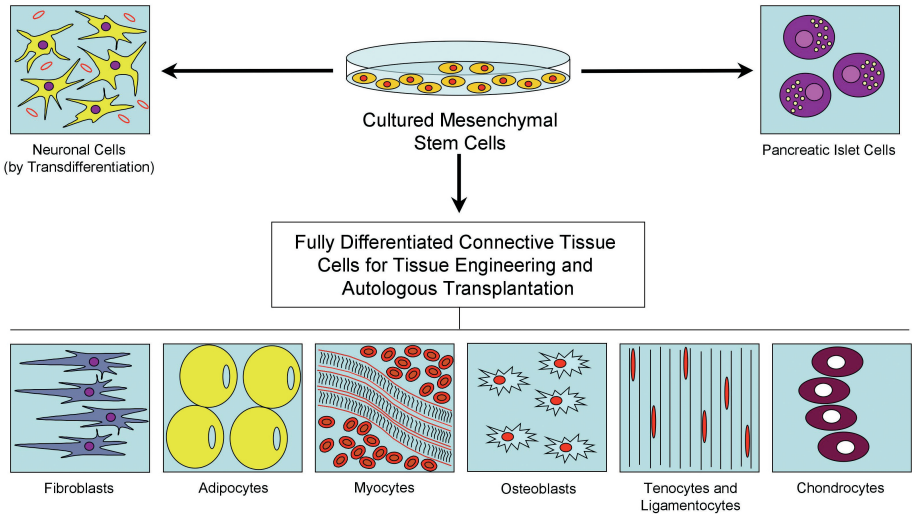

Fig. 4. Applications of cultured mesenchymal stem cells. Mesenchymal stem cells have the capacity to differentiate into connective tissue and musculoskeletal cells for tissue engineering, autologous implantation/transplantation and regenerative medicine. This process involves commitment, lineage progression, differentiation and maturation. Mesenchymal stem cells have recently been shown to differentiate into pancreatic islet cells. Mesenchymal stem cells may also have the capacity to give rise to neuronal cells through a process known as transdifferentiation.

1999; Majumdar et al., 2000; Short et al., 2003; Kramer et al., 2004; Otto and Rao, 2004) (Fig. 4).

MSCs represent 2-3% of the total mononuclear cells in bone marrow and can be isolated and expanded for several passages without loosing their ability to differentiate (Pittenger et al., 1999). In monolayer culture they show a typical colony forming fibroblast-like morphology which they maintain throughout many passages and they express several adhesion molecules found also in mesenchymal, endothelial and epithelial cells (Conget and Minguell, 1999). Extraction of bone marrow is easily obtained by bone marrow aspirates (Caplan et al., 1997). The bone marrow is immediately diluted in 3% citric acid or heparin and, after transport to a cell culture laboratory, the stem cells are isolated mainly through density gradient centrifugation, magnetic bead sorting or FACS analysis. The International Society for Cellular Therapy has listed the main factors required for a cell to be regarded as a mesenchymal stem cell in a position statement (Dominici et al., 2006). Mesenchymal stem cells are characterized through their adhesion potential in monolayer culture and through their differentiation potential into chondrocytes, osteocytes and adipocytes *in vitro* (Fig. 5). Furthermore, the International Society for Cellular Therapy has listed several markers that cells should exhibit or lack in order to be classified as MSCs. Markers that MSCs should exhibit include CD105+, CD73+ and CD90+, whereas MSCs should lack CD45-, CD34- and several other hematopoietic stem cell markers (Dominici et al., 2006).

Other sources for the isolation of mesenchymal stem cells include umbilical cord blood (Bieback et al., 2004; Kogler et al., 2004), adipose tissue (Zuk et al., 2002; Kern et al., 2006), synovial cells (De Bari et al., 2001; Sakaguchi et al., 2005; Koga et al., 2007) and peripheral blood (Huss et al., 2000; Zvaifler et al., 2000; Ukai et al., 2007). Adipose tissue in animals seems to be a good and plentiful source of MSCs (Qu et al., 2007; Yamamoto et al., 2007). Umbilical cord blood has proven to be a good source of MSCs. Human cord blood non-haematopoietic stem cells have been differentiated into multiple cell types such as endothelial cells, neurons, smooth muscle cells, adipocytes, chondroblasts and osteoblasts (Aoki et al., 2004; Bieback et al., 2004; Kogler et al., 2004; Watt and Contreras, 2005; Qu et al., 2007; Yamamoto et al., 2007). There has been some discussion and debate about the routine collection and deep freezing of umbilical cord blood from babies in case the need arises in later life. Umbilical cord blood derived MSCs are already commercially available for horses and humans. The equine sporting industry is hoping for regeneration of cartilage and tendon injuries with umbilical cord blood derived MSCs (Koch et al., 2007).

Adipose tissue is another alternative stem cell source that can be obtained by less invasive methods and in larger quantities than bone marrow. It has been demonstrated that adipose tissue contains stem cells

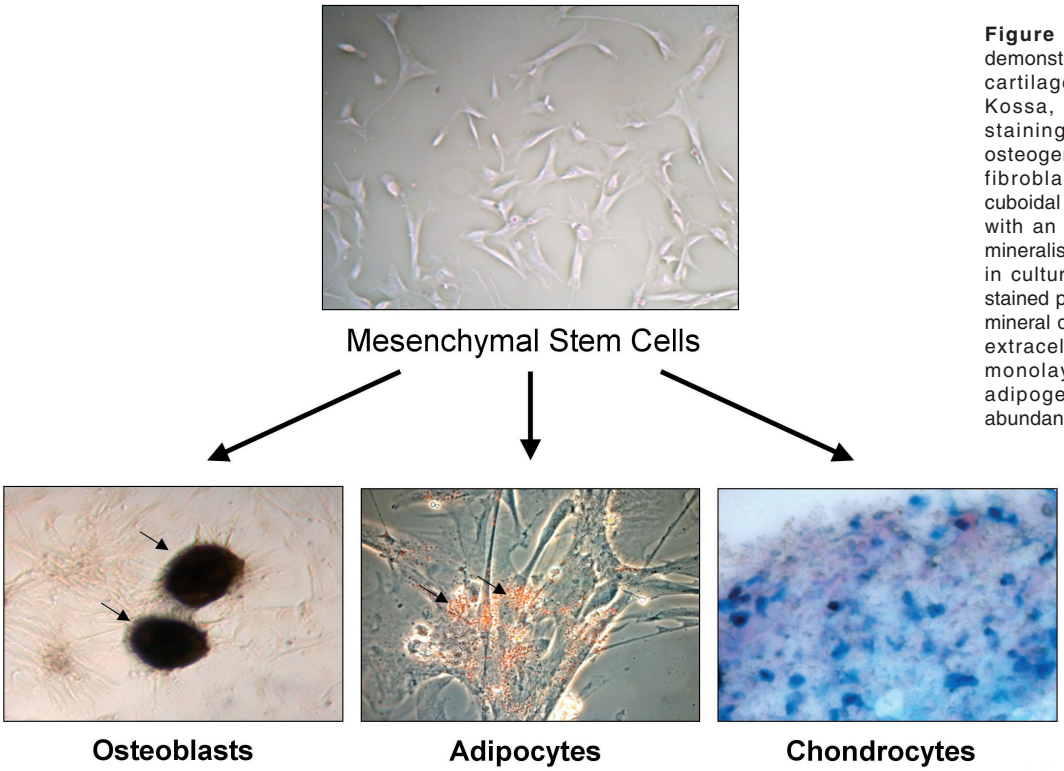

Figure 5: Light microscopic demonstration of osteoid, adipose and cartilage tissue formation with von Kossa, Oil Red O and Alcian blue staining. Cells changed with the osteogenic induction medium from a fibroblastic appearance to a more cuboidal appearance, were surrounded with an abundant matrix and formed mineralised nodules. After three weeks in culture, the stimulated cells were stained positive with von Kossa stain for mineral deposition in their newly formed extracellular matrix (arrows). In the monolayer cultures treated with adipogenic induction media an abundance of vacuoles were observed in the cells. Oil Red O staining for lipids revealed that these vacuoles indeed contain neutral lipids (arrows). After 21 days the high density cultures treated with chondrogenic induction medium (i.e. TGFß-1 and dexamethasone) were intensely stained with Alcian blue revealing a high content of cartilage specific proteoglycans. B, x 10; C, D, x 40

similar to bone marrow-MSCs, which are termed processed lipoaspirate (PLA) cells (Zuk et al., 2002). These cells can be isolated from cosmetic liposuctions in large numbers and grown easily under standard tissue culture conditions. The multilineage differentiation capacity of PLA cells has been confirmed. It has also been observed that human adipose tissue yields higher amounts of MSCs then bone marrow or umbilical cord blood (Kern et al., 2006). However, in humans it seems that bone marrow obtained MSCs have superior chondroprogenitor capacities compared to adipose tissue derived MSCs (Im et al., 2005).

Chondrogenic stem cell differentiation

Current attempts at chondrogenic differentiation of MSCs are based on the knowledge of chondrogenic development, cartilage homeostasis and function. The use of mesenchymal stem cells for articular cartilage repair is based on the awareness that *in vivo*, during embryogenesis, limb formation occurs through the condensation of mesenchymal cells which then differentiate to the chondral pre-skeleton and form the cartilage covering the articulating surface of the joint.

Chondrogenesis *in vitro* requires two major components: firstly a 3-dimensional environment (Fig. 6) and secondly the addition of various combinations of growth factors to stimulate chondrogenic signaling pathways in the MSCs. The micromass pellet culture as described by Pittenger and co-workers is the most frequently used system (Pittenger et al., 1999). Here, 250,000 to 500,000 MSCs are centrifuged in a conical tube and then incubated for various time periods ranging from 14 to 21 days at 37°C in a humidified atmosphere. After one day in culture, the cells aggregate and form a round cell pellet. Other 3-dimensional culture methods for cartilage formation include high density bridge cultures (Fig. 6) and alginate beads cultures (Shakibaei and De Souza, 1997; Lange et al., 2005).

Since the discovery that MSCs can be directed towards the chondrogenic lineage, many attempts have been made towards cartilage tissue engineering (Caplan and Goldberg, 1999; Magne et al., 2005). It is well known that MSCs can be stimulated towards the chondrogenic lineage with a variety and various combinations of soluble factors (Fig. 7). These include TGF-ß1, IGF-1, dexamethasone, the family of bone morphogenic proteins (BMPs) and fibroblast growth factor (FGF) (Grigoriadis et al., 1988; Denker et al., 1999; Pittenger et al., 1999; Nixon et al., 2000; Carlberg et al., 2001; Tsutsumi et al., 2001; Nakayama et al., 2003).

TGF-ß1 is the most commonly used growth factor for *in vitro* chondrogenic differentiation of MSCs (Caplan, 1991; Johnstone et al., 1998; Pittenger et al., 1999). TGF-ß1s mechanism of action has not yet been fully elucidated. The TGF-ß1 and Wnt signaling pathways enhance proliferation of MSCs and simultaneously inhibit their differentiation towards osteoblasts and adipocytes (Zhou et al., 2004; Jian et al., 2006). Longobardi and co-workers showed that a part of its mechanism is mediated by ERK1/2 MAPKinase signaling pathway (Longobardi et al., 2006). Thereby they could show that ERK1/2 mediates mitogenic properties of TGF-ß, however collagen type II production is independent from this. The observation of Jaiswal and colleagues that activation of ERK1/2 plays an important role in osteogenesis of MSC and that the inhibition of the MAPKinase pathway leads to adipogenesis agrees with these findings (Jaiswal et al., 2000). Longobardi et al. also showed that IGF-1 independently from TGF-ß can regulate chondrogenesis in MSCs and that the signal transduction pathway of IGF-1 only partly runs via the MAPKinase pathway (Longobardi et al., 2006). They postulate a synergism between TGF-ß and IGF-1, which positively stimulates chondrogenesis in the MSCs. Expression of the chonodrogenic specific transcription factor Sox9, the quantities of collagen type II and the cartilage specific proteoglycans (CSPG) in MSCs stimulated both with TGF-ß and IGF-1 was comparable to that of mature adult chondrocytes (Longobardi et al., 2006). Derfoul and co-investigators studied the mechanism of action of dexamethasone, a synthetic glucocorticoid, and showed that in MSCs chondrogenesis was directly stimulated via the glucocorticoid receptor. Additionally it enhances TGF-ß mediated collagen type II and CSPG, i.e. aggrecan, production (Derfoul et al., 2006). Furthermore it has been described that MSCs from various animals such as horses, rabbits and cattle could be differentiated towards chondrocytes with dexamethasone as the main medium supplement (Grigoriadis et al., 1988; Johnstone et al., 1998; Bosnakovski et al., 2005). Proteins belonging to the BMP family of proteins can also stimulate chondrogenesis in MSCs *in vitro* (Luyten et al., 1992; Sailor et al., 1996; Chubinskaya and Kuettner, 2003; Schmitt et al., 2003; Indrawattana et al., 2004; Knippenberg et al., 2006). Stimulation of MSCs with BMP-7 has been shown to lead to chondrogenesis (Knippenberg et al., 2006). Therefore, BMP-7 seems to

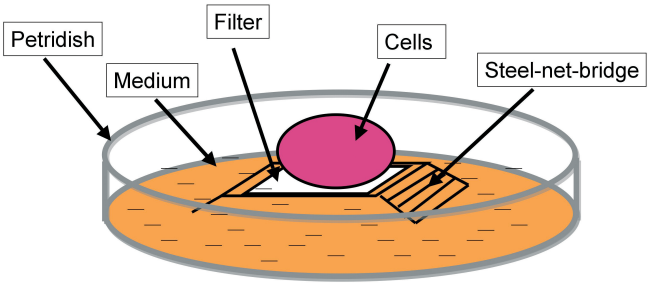

Fig. 6. Schematic of the three dimensional system for high density cultures. A nitrocellulose filter is placed on a steel-net-bridge and cells are cultured on the filter. Cell culture medium reaches the filter-medium interface, nurturing cells through diffusion, thus mimicking an environment similar to that found *in vivo*.

suppress osteogenic differentiation pathways in the MSCs. Contrary to this, sole stimulation with BMP-2 seems to stimulate osteogenesis (Knippenberg et al., 2006), while a combination of BMP-2 and TGFß-3 leads to chondrogenesis (Schmitt et al., 2003). Chondrogenesis, through combination treatment of BMP-6 and TGFß-3, has also been described (Indrawattana et al., 2004). It is well known that BMP-2 cooperates with the Wnt signal transduction pathway. BMP-2 can upregulate Wnt3a, leading to accumulation of ß-Catenin and the subsequent induction of Sox9 and chondrogenesis (Fischer et al., 2002a,b). Upregulation of ß-Catenin is essential to commit a cell to the chondrogenic lineage (Day et al., 2005; Hill et al., 2005). However, in mature adult chondrocytes, ß-Catenin can also stimulate chondrogenic hypertrophy and ossification (Kitagaki et al., 2003; Tamamura et al., 2005). This is a logical step since although articular cartilage chondrocytes normally stay at a mature state, in growing adolescents the chondrocytes become hypertrophic, produce alkaline phosphatase and collagen type X and are then eventually reabsorbed while new bone is formed at the site of the endochondral bone growth plate. Another soluble factor used for chondrogenic differentiation of MSCs is fibroblast growth factor 2 (FGF-2). MSCs that have been stimulated with FGF-2 in monolayer culture after being transferred to a 3-dimensional environment, produce more CSPG and cells with a round chondrogenic phenotype (Solchaga et al., 2005a; Chiou et al., 2006). Chondrogenic induction hereby seems to be mediated via the MAPKinase signaling pathway (Murakami et al., 2000). Treatment with FGF-2 has been shown to significantly enhance Sox9 expression and that this is mediated via the MAPKinase signal transduction pathway (Murakami et al., 2000). It is well known that the MAPKinase pathway plays a pivotal role in differentiation, development of the chondrogenic phenotype and specific function of chondrocytes (Shakibaei and Merker, 1999; Shukunami et al., 2001). It has recently been shown that ERK1/2 even physically interacts with Sox9 (Shakibaei et al., 2006).

Mesenchymal stem cells and cartilage repair

The simplest approach of cartilage repair using MSCs can be done analogous to the ACT method. After expanding the MSCs *in vitro* they are injected into a cartilage defect. MSCs are hereby frequently combined with a soluble scaffold to ensure they remain in the cartilage defect similar to the fibrin glue or the periosteal flap used in classic ACT. The results have been ambiguous. Some have reported the formation of new cartilage, whereas others have shown degradation and fragmentation of the MSCs. Most studies are still in the experimental trial phase and have been performed using animal models, such as rabbit (Im et al., 2001) and goat (Quintavalla et al., 2002; Murphy et al., 2003). In

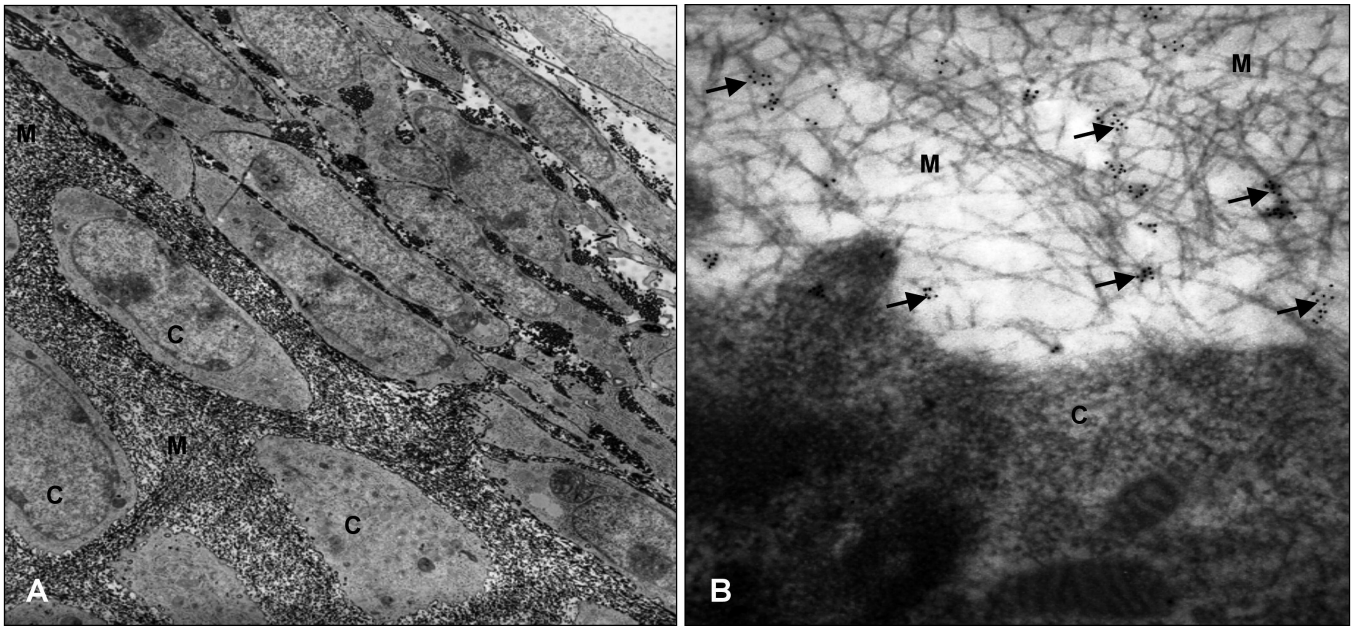

Fig. 7. A. Electron microscopic demonstration of MSCs induced with chondrogenic medium in high-density culture. After 14 days nodules with typical round or oval chondrocytes (C) with small processes embedded in a network of extracellular matrix fibrils (M) could be observed. These nodules, typical for cartilage formation, were surrounded by a layer of flattened, fibroblast-like cells embedded in fibrils of thick extracellular matrix. x 5000. **B.** Transmission immunoelectron microscopic images of high-density MSC cultures induced with chondrogenic induction medium. Chondrogenic induced cultures were immunogold labelled with anti-cartilage-specific proteoglycan antibodies. Anti-cartilage specific proteoglycan labelled gold particles formed clusters which were distributed irregularly in the extracellular matrix (arrows). x 20,000

rabbits, positive results regarding new cartilage formation have been reported after MSC administration into the defect (Im et al., 2001). Here, immunohistochemical staining of the newly formed cartilage was more intense for type-II collagen in the matrix and higher amounts of mRNA for type-II collagen were detected compared to the control group. In a study using goats subjected to total removal of the medial meniscus, there was evidence of marked regeneration of the medial meniscus after MSC injection, as well as detection of the implanted cells in the newly formed tissue (Murphy et al., 2003). Furthermore, degeneration of the articular cartilage, subchondral bone remodeling, osteophyte formation and subchondral sclerosis were reduced in cell-treated joints compared to joints treated with the vehicle alone. Contrary to this, in another goat cartilage defect model, two weeks post-implantation a gradual loss of implanted cells in the defect as well as fragments of gelatin sponge containing labelled MSC in deep marrow spaces was observed, indicating fragmentation, dislodgement and passive migration (Quintavalla et al., 2002). In other studies MSCs have been implanted after differentiation towards chondrocytes or on scaffolds with successful formation of cartilage-like tissue in parts of the defect (Wakitani et al., 1994; Meinel et al., 2004; Liu et al., 2006).

Clinical trials of human patients undergoing cartilage Repair with MSCs

Although a number of animal studies have been performed using MSCs for articular cartilage repair, until now only a few clinical trials have been performed on human patients (Wakitani et al., 2002). This is, as indicated above, mainly due to the varying results that the treatment of cartilage defects with MSCs has achieved in animal models. Recently however, a group from Japan performed a study on 24 patients diagnosed with OA (Wakitani et al., 2002). In this study, autologous MSCs were obtained from the patients' bone marrow and expanded in monolayer culture. After obtaining sufficient cell numbers, MSCs were seeded onto a collagen type I membrane and transplanted into the cartilage defect. 12 patients served as the control group and received cell free implants. Histological evaluation of biopsies taken after two years showed significantly higher hyaline cartilage formation in the treated compared to the untreated group. However, there was no way of tracking the implanted MSCs for this long time period so it remains unclear whether the newly formed tissue was consisting of the implanted MSCs.

Problems with tissue engineering cartilage from MSCs

MSCs may represent a useful cell source as an alternative approach to the treatment of cartilage defects due to their availability in relatively high numbers and the easy ways to obtain them. However, a limiting factor is that in the cartilage resulting from MSCs, markers connected with hypertrophic chondrocyte differentiation such as alkaline phosphatase and collagen type X are up-regulated and growth factors are needed for chondrogenic induction (Johnstone et al., 1998). MSCs that differentiate towards the chondrogenic lineage *in vitro* do not express a stable chondrogenic phenotype. Collagen type X is considerably up-regulated in 3-dimensional culture and detectable around day seven with RT-PCR (Johnstone et al., 1998; Barry et al., 2001) and around day 14 with immunohistochemistry (Yoo et al., 1998). In healthy mature chondrocytes and in engineered cartilage from mature chondrocytes, collagen type X is either not present or is only marginally expressed (Riesle et al., 1998; Tallheden et al., 2004; Zhang et al., 2004). As collagen type X makes up 45% of the collagen produced in hypertrophic chondrocytes, it is considered an important marker of endochondral bone formation (Gibson and Flint, 1985; Shen, 2005).

MSCs that differentiate towards the chondrogenic lineage exhibit elevated levels of alkaline phosphatase expression and activity. The induction of chondrogenesis in MSCs is accompanied by an increase of alkaline phosphatase activity around day 7 reaching a peak around day 14. Contrary to this, in normal mature articular chondrocytes from the superficial and the middle zone of the joint surface, no alkaline phosphatase activity can be detected. Alkaline phosphatase activity has been described in hypertrophic chondrocytes in the calcified zone, in endochondral ossification centers, the growth plate and bone (Henson et al., 1995; Miao and Scutt, 2002).

Conclusions and future perspectives

The ultimate goal of clinicians and scientists involved in cartilage and OA research is achieving better articular cartilage repair and eliminating or significantly reducing pain and inflammation while restoring a mechanically functional repair tissue. However, the scientific and clinical evidence provided thus far suggests that we are still a long way from being able to create functionally competent hyaline cartilage with a zonal architecture similar to that found in native articular cartilage. Although the research done in the last two decades has narrowed the gap to achieving this goal, there is still a long way to go. Nevertheless, the pace of progress has been significantly enhanced with the advent of tissue engineering and stem cell technologies. No matter which approach is favored, the outcome is still and will remain a more effective and robust hyaline cartilage repair. ACT has established itself in clinical medicine and has become a widely accepted method for treating cartilage defects in OA.

Tissue engineering is an integral part of regenerative medicine that seeks to address the urgent need for replacement tissues. The delivery of phenotypically stable cells in 3-dimensional scaffolds to promote healing for repair, replacement or regeneration of tissues

is a key aspect of connective tissue engineering. The seamless integration of basic biology and materials engineering is a defining characteristic of tissue engineering and regenerative medicine. However, it is critical is that any bioengineered material has a 3-dimensional structure that is designed to meet specific physical requirements of the original tissue and promote cell and tissue integration. This article has focused on ACT, MSCs, cartilage tissue engineering and has critically evaluated the strengths and limitations of existing techniques for articular cartilage repair and regeneration. Future studies will need to focus on the intricacies of the unique cell biology of MSCs and the biochemical signal transduction pathways involved in maintaining and enhancing chondrogenic differentiation. This knowledge will enable tissue engineers to create more durable biomaterials capable of replacing cartilage defects. Understanding the biology of MSCs and their interaction with engineered 3-dimensional scaffolds will enable a variety of connective tissues including cartilage, bone, tendon and ligament to be generated in the laboratory for applications in regenerative medicine and autologous transplantation.

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