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Mesenchymal stem cells as a potential pool for cartilage tissue engineering

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Contents

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Introduction

In modern medicine the terms ''regenerative medicine'' and ''Tissue Engineering'' have become key words, envisioning our ability to grow and to engineer functional tissue *in vitro* that will enable us to repair and substitute damaged or degenerated tissue *in vivo* (Caplan and Goldberg, 1999; Solchaga et al., 2001). Tissue engineering can be defined as the art of reconstructing mammalian tissue, both structurally and functionally (Hollander et al., 2006), whereas this reconstruction process may be performed entirely *in vitro*, with mature tissue being transplanted, or partially *in vitro* followed by a maturation process after transplantation *in vivo*. Here we attempt to imitate natural processes in our bodies, which, in order to survive, must be able to repair and regenerate damaged tissues by replacing them with progenitor cells that have the capacity to differentiate into the specialized cell type (Tallheden et al., 2006). Modern medicine has greatly improved our abilities in the fields of diagnosis, prevention and treatment of a range of infectious, metabolic, neurodegenerative, cardiovascular, respiratory and cancerous diseases. New understandings of embryonic development and new technical skills have led to the therapeutic approach of repairing connective tissue through tissue engineering, thus opening an entirely new starting point for cartilage repair (Cancedda et al., 2003; Caplan, 1991, 2000; Caplan et al., 1997; Short et al., 2003). The need for effective cartilage repair strategies has been rising continuously, as the increase in life expectancy in humans also leads to a major increase in rheumatoid arthritis and osteoarthritis (OA). It is estimated that more than 39 million people in the European Union and over 20 million Americans have OA and it is anticipated that by the year 2020, these numbers will have doubled. In Germany, OA has major economic consequences, creating direct and indirect costs of 8 billion Euros per annum.[1] As existing pharmaceuticals (steroids and non-steriodal anti-inflammatory drugs) are unsatisfactory as they only treat the symptoms of OA by reducing pain and inflammation, these numbers make it clear that new effective treatments for OA are of vital importance.

This article will present selected methodologies for cartilage repair and the potential usefulness of mesenchymal stem cells (MSCs) and critically evaluate their strengths and limitations in articular cartilage regeneration and tissue engineering.

Challenges in connective tissue engineering

Tissue engineering has evolved as a new field, promising *in vitro* construction of whole transplantable tissue. Basically, three main components are required for successful tissue engineering: Firstly, a scaffold providing an adequate three-dimensional surrounding. Secondly, appropriate cells which are able to differentiate and maintain the specific cell phenotype. Thirdly, the addition of the right bioactive substances such as growth factors, cytokines or hormones as a suitable stimulus for specific lineage differentiation of the cells (Kuo et al., 2006) (Figure 1). Connective tissue injuries of the bone, tendon and cartilage represent a large fraction of trauma medicine. Although bone has a high turnover rate, is vascularised and callous formation happens rather quickly, large bone defects resulting from massive traumata, tumors or metabolic and degenerative diseases have a limited capacity for self-repair and require special treatment strategies (Kajiwara et al., 2005; Krampera et al., 2006). Although osteochondral grafts have been successfully used for several decades (Czitrom et al., 1986; Marco et al., 1993; Meyers et al., 1983), grafting is often related to donor-site morbidity, increased risk of infection from allografts, immunocompatibility, implant rejection and necrosis (Hangody and Fules, 2003). Also tendon injuries, often the consequence of

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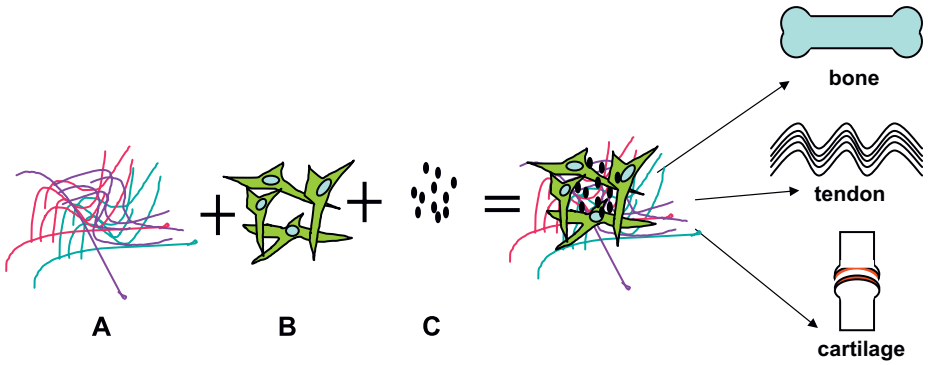

Figure 1. The principle of tissue engineering. The concept of tissue engineering comprises three main factors: Firstly, a compatible biomaterial (A), secondly adequate cells, such as stem cells (B) and thirdly the addition of specific bioactive substances (C) enhancing appropriate cell differentiation and specific tissue formation such as bone, tendon and cartilage.

recurrent micro- or macro-traumata or the side effect of antibiotic treatment with gyrase inhibitors such a chinolones (Bosch, 2000; Hankemeier et al., 2007; Sendzik et al., 2005; Shakibaei et al., 2000, 2001a; Shakibaei and Stahlmann, 2001, 2003) present another major clinical problem, as tendon, like cartilage, is a bradytroph tissue with slow and very limited regenerative capacities (Beris et al., 2005; Moller et al., 2000; Schulze-Tanzil et al., 2004a; Ahmed et al., 1998). Furthermore, the tendon also contains very few cells, the tenocytes, which produce a highly specialized extracellular matrix (ECM) (Canty and Kadler, 2002; Kannus, 2000; Rees et al., 2000). As with bone, the present regeneration strategies for tendon repair evolve around allo- and autografting of tendon; however, recently, treatments have been expanded by novel tissue-engineering approaches using bio-compatible and bio-degradable scaffolds in combination with tenocytes or MSCs (Krampera et al., 2006). The specific characteristics of articular cartilage are mainly due to the special construction of its ECM. The ECM is produced by the cartilage cells, the chondrocytes, which make up less than 5% of the tissue's total three-dimensional volume. A particular feature is that the cells do not have any direct cell-to-cell contact with each other and each cell has to be regarded as an individual functional unit responsible for maintaining the ECM in its immediate surrounding through balanced and tightly regulated anabolic and catabolic activities. The ECM produced by the chondrocytes is specific for hyaline cartilage and 40–50% consists of collagens (90% of which is collagen type II) and 20–25% consists of different proteoglycans (aggrecan, decorin, biglycan and fibromodulin) (Kuettner, 1992). Cartilage develops during embryogenesis through condensation and subsequent differentiation of MSCs. During this process the cells start to synthesise cartilage matrix-specific proteoglycans and collagen type II (Cancedda et al., 1995). In adults, articular cartilage chondrocytes retain a mature state; however, in adolescents during enchondral bone development, at the site of the growth plate the chondrocytes become hypertrophic, produce alkaline phosphatase and collagen type X and are eventually reabsorbed while new bone is formed (Cancedda et al., 1995; Kuettner, 1992). For scientists and medical doctors cartilage repair presents a major challenge because of its unique construction. Cartilage itself lacks both vascularisation and innervation. Healing processes are therefore slow and the resulting scar tissue most often lacks the necessary mechanical properties and physical durability of the original articular cartilage (Cancedda et al., 2003; Vachon et al., 1986). This scar tissue cannot withstand the physiological strain and the result is further degeneration of the cartilage, continued decline in joint function, inflammation, restricted joint movement and deformity. The main symptoms of OA are therefore well known: pain, stiffness and swelling of the joints. Advanced OA leads to further instability, putting stress on the ligaments and tissues surrounding the joints. Currently, more than 200 diseases that affect the joints are summarized under the term OA. Established treatments for OA include mainly preventive measures such as weight control, exercise or treatment of underlying metabolic diseases. Recently, neutraceuticals, which contain polyphenolics, have also been discussed for OA treatment (Csaki et al., 2008; Khanna et al., 2007; Shakibaei et al., 2007a, b). Conservative surgical techniques used to achieve clinical cartilage regeneration (Hunziker, 2002) such as the micro-fracture method (Steadman et al., 1999; Sledge, 2001) and the mosaic-plastic method (Hangody et al., 2001a, b) involve autologous

grafting of chondral or osteochondral fragments. However, the results of trials on OA patients have been unsatisfactory (Quinn et al., 1998a, b; Wohl et al., 1998).

Cartilage tissue engineering

One of the best-known current regenerative medicine strategies to achieve cartilage defect repair is the Autologous Chondrocyte Transplantation (ACT) method. The ACT method was introduced in the 1980s by a Swedish group as a novel clinical treatment for articular cartilage repair to solve the problem of progressive degeneration in OA joints (Brittberg, 1999). Following the basic principles of repair, a cartilage defect was filled with autologous chondrocytes (i.e. derived from is the same patient). The idea was that, by introduction of the chondrocytes in single-cell suspension into the defect, cell condensation would be triggered, imitating the condensation phase in early embryonic development. This condensation phase in turn would provide the chondrocytes with an adequate stimulus to synthesis new cartilage-specific matrix and result in regeneration of articular cartilage tissue in the cartilage defect. In a biopsy a 150–300 mg sample of cartilaginous tissue is removed in an initial surgical procedure from a non-weight-bearing area of the joint, for example in the knee from the supromedial edge of the femoral condyle, and expanded as a cell culture *in vitro* until enough cells are obtained for defect filling. In the second surgical procedure, these cells are then re-implanted into the cartilage defect. To secure the chondrocytes at the implanted side and prevent them from floating away, a periosteal flap is further sewed over the defect (Brittberg et al., 1994, 1996). In several animal studies ACT has shown excellent outcomes (Breinan et al., 1997; Brittberg et al., 1996; Dell'Accio et al., 2003; Grande et al., 1989; Lee et al., 2003; Rahfoth et al., 1998). In human patients ACT has been performed on over 12,000 patients worldwide (Peterson et al., 2000). Results after 3–9 years are very encouraging, with a significant reduction in pain reported in treated patients (Peterson et al., 2002), although repair of the defect is not uniform in all areas of the joint (Brittberg et al., 1994; Peterson et al., 2000).

Although ACT has been in use for some time, it still faces several major challenges, including donor-site morbidity, chondrocyte de-differentiation during *in vitro* culture and fibrocartilage formation after cell implantation instead of defect healing (40% of ACTs show evidence of chondrocytes hypertrophy) (Dell'Accio et al., 2003; Brittberg et al., 2003; Schulze-Tanzil et al., 2002, 2004b; Brittberg, 1999). Especially, chondrocyte de-differentiation during monolayer culture poses problems. *In vivo*, chondrocytes are embedded in a well-structured ECM, helping them to maintain their function and vitality (Shakibaei et al., 1993, 1997; Shakibaei and Merker, 1999). Monolayer culture, in which chondrocytes are forced to give up their chondrogenic phenotype and the absence of their specific ECM, leads to a shift in the chondrocytes from the production of cartilage-specific proteins, such as collagen type II, to non-specific proteins, such as collagen type I (von der Mark, 1980; von der Mark et al., 1977; Marlovits et al., 2004). After re-implantation these de-differentiated chondrocytes continue to produce unspecific matrix components leading to a more fibrocartilage repair tissue that lacks the biomechanical properties and the resilience of articular cartilage. Up to the fourth passage *in vitro* chondrocytes can spontaneously re-differentiate in a three-dimensional environment (Schulze-Tanzil et al., 2002; Shakibaei et al., 2006). Also, bioactive stimuli such as insulin-like growth factor I (IGF-I) and the transforming growth factor beta (TGF-β) have been shown to prolong and reconstitute the re-differentiation capacity of monolayer-expanded chondrocytes (Barbero et al., 2003; Hunziker, 2001; Jenniskens et al., 2006; Shakibaei et al., 2006).

Next-generation ACT

As the classical ACT method has shown flaws, refined approaches to promote chondrocyte re-differentiation for effective cartilage repair are on trial, combining chondrocytes with a great variety of carrier systems (scaffolds) or biomaterials (Marlovits et al., 2006). The so-called "second-generation ACT" uses a biomaterial membrane (such as the bilayer collagen type I/type III membrane Chondro-Gide™) instead of the periostal flap to secure the chondrocytes in the defect. The aim is to reduce a periostal reaction connected with the periostal flap usage such as periosteal hypertrophy. In the so-called "third-generation ACT" a three-dimensional environment is created *in vitro* with a scaffold; this is loaded with chondrocytes and this neo-cartilaginous tissue-engineered construct re-implanted. Although materials vary greatly from producer to producer (Hunziker, 2002), in general, scaffold material has to be structurally and mechanically stable, reabsorbable and non-toxic for the cells (Tuli et al., 2003). Some of the most frequently used materials

are polylactide-*co*-glycolide-based (Mercier et al., 2005), hyaloron-based (Solchaga et al., 2005b) or atelocollagen-based (Ochi et al., 2002). *In vitro* and *in vivo* studies on animals (Wakitani et al., 1998; Solchaga et al., 2005b) and humans (Ochi et al., 2002; Zheng et al., 2007) had quite positive results with up to 75% hyaline cartilage formation after 6 months (Zheng et al., 2007). However, severe signs of hypertrophy and partial ossification of the implants could also be observed (Ochi et al., 2002). As scaffolds may pose additional problems depending on their composition, scaffold-free techniques (Kelm and Fussenegger, 2004; Marlovits et al., 2003) have also been implemented in animal models (Mainil-Varlet et al., 2001; Barnewitz et al., 2003). To enhance tissue integration, several biological substances such as fibrin glue or collagen cross-linkers are on trial, however, up to now without sweeping results (Ahsan et al., 1999; Jurgensen et al., 1997; Grande and Pitman, 1988).

As cartilage lesions are generally large and unconfined and do not provide an appropriate environment for chondrocytes to be retained long enough to elaborate an ECM, problems also remain in second- and third-generation ACT, such as the poor integration of the repair tissue into the surrounding cartilage (Ahsan et al., 1999; Hunziker, 2001, 2002), so that the required size of the neocartilaginous construct to fill up the cartilage defect frequently cannot be achieved (Hunziker, 2002) and the loss of the chondrocytes chondrogenic phenotype and re-differentiation potential resulting in chondrocytes being incapable of cartilage production after *in vivo* implantation (Schulze-Tanzil et al., 2002, 2004b; Shakibaei et al., 1999). Therefore, successful repair of cartilage lesions is only likely to be achieved when three-dimensional cartilage implants can be generated that have enough ECM for fixation within the joint.

To achieve this goal a precise knowledge of the biochemical and molecular signaling pathways activated and involved in chondrogenesis is therefore vitally important. A major signaling pathway involved in activation of the chondrogenic differentiation of chondrocytes is the MAPKinase pathway (Schulze-Tanzil et al., 2004b; Shakibaei et al., 2001b), which stimulates the specific chondrogenic transcription factor Sox9. Growth factors such as the insulin like growth factor-I or the transforming growth factor-β1 have been shown to stimulate the MAPKinase pathway through activation of the adaptor protein Shc (Src homology protein) which in turn activates the MAPKinase members ERK 1/2 (extracellular regulated kinase 1/2) (Shakibaei et al., 2006). Monolayer studies have already shown that *in vitro* stimulation with these growth factors prolongs the chondrocyte re-differentiation potential suggesting that growth factor treatment may indeed be a new approach for ACT (Shakibaei et al., 2006).

MSCs for cartilage regeneration

As stated above, obtaining vital and differentiated chondrocytes presents one of the major challenges for successful ACT. Not only that each biopsy presents an additional trauma to an already damaged joint cartilage, but the expansion phase the chondrocytes have to undergo *in vitro* leads to rapid cell de-differentiation with a loss of their chondrogenic potential (von der Mark et al., 1977; Darling and Athanasiou, 2005; Shakibaei et al., 1997). Although re-differentiation of these cells has been shown *in vitro* with (Barbero et al., 2003; Jakob et al., 2001) and without (Anderer and Libera, 2002) the addition of growth factors such as TGF-β, an alternative, easily obtainable cell source with stable chondrogenic potential becomes necessary. As other cartilage sources such as nasal and rib cartilage have not been fully assessed for their ability to repair articular cartilage (Naumann et al., 2002), an undifferentiated progenitor cell that possesses multilineage differentiation potential and is present everywhere in the body would be ideal for tissue engineering. Here, MSCs present themselves as a promising cell source for the regeneration of cartilage as they possess chondrogenic differentiation potential, are easily obtainable in high numbers and expandable *in vitro* without losing their differentiation potential (Caplan and Goldberg, 1999; Pittenger, 2008).

Sources, isolation and characterization of MSCs

The term stem cell is used to denote an unspecialized progenitor cell residing in niches in various organs and tissues from which it can be recruited to replenish specific tissue cells when they die (Caplan and Dennis, 2006; Fuchs et al., 2004). Stem cells are not only found in the embryo but also in the fetus and in the adult individual. The acquisition of stem cells for tissue engineering from the fetal or adult individual has the advantage of avoiding possible immunologic responses connected with allogenic cell transplantation. Furthermore, the use of embryonic stem cells presents ethical considerations that are redundant in adult or fetal stem cells. The earliest stem cells to be identified belong to the hematopoetic lineage and were

isolated from the bone marrow. Today, stem cells have been discovered in almost all organs including peripheral blood, bone marrow, muscle, fat, pancreas, skin, neuronal system and others (Till and McCulloch, 1980; Wickham et al., 2003; Zuk et al., 2001; Bottai et al., 2003; Alexanian and Sieber-Blum, 2003; Williams et al., 1999; Caplan, 1991; Cancedda et al., 2003). MSCs have been isolated from bone marrow (Caplan et al., 1997; Friedenstein et al., 1987, 1966), umbilical cord blood (Bieback et al., 2004; Kogler et al., 2004), adipose tissue (Zuk et al., 2002; Kern et al., 2006) and peripheral blood (Huss et al., 2000; Zvaifler et al., 2000; Ukai et al., 2007; Koerner et al., 2006; Giovannini et al., 2008). In the sixties, Friedenstein et al. (1966) had already described the presence, in bone marrow, not only of hematopoietic stem cells but also of MSCs capable of osteogenesis *in vitro*. Today bone-marrow-derived MSCs have been differentiated into various specific cell lineages (Pittenger et al., 1999; Short et al., 2003; Caplan, 1991; Otto and Rao, 2004; Majumdar et al., 2000; Kramer et al., 2004; Conget and Minguell, 1999; Fortier et al., 1998; Carlberg et al., 2001; Csaki et al., 2007). MSCs present approximately 2–3% of the total nuclear cell fraction in the bone marrow. They can easily be isolated through bone marrow aspiration and expanded over several passages without losing their differentiation potential (Caplan, 1991; Csaki et al., 2007; Pittenger et al., 1999). After obtaining the bone marrow aspirate, for example in humans and canines from the iliac crest or in horses often from the sternum, it should be immediately diluted in 3% citric acid or heparin to prevent blood coagulation. After arrival in the cell-culture laboratory, the stem cells are separated from the remaining bone marrow through density-gradient centrifugation with Ficoll or Percol (for example from Biochrom, Germany) and plating of the nuclear fraction, through plating the entire bone marrow after erythrolysis, through separating the MSCs by magnetic cell sorting or by FACS analysis (Caplan, 1991; Lange et al., 2005; Lee et al., 2004; Martin et al., 2002). One of the main characteristics of stem cells is their potential to adhere onto plastic during *in vitro* conditions (Figure 2). *In vitro* MSCs have a fibroblast-like morphology which they maintain during extended passaging. Furthermore, they express several adhesion molecules also found on mesenchymal, endothelial and epithelial cells (Conget and Minguell, 1999). 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). MSCs are characterized by their adhesion potential in monolayer culture and their differentiation potential into chondrocytes, osteocytes and adipocytes *in vitro* (Figure 3). Furthermore, the International Society for Cellular Therapy has listed

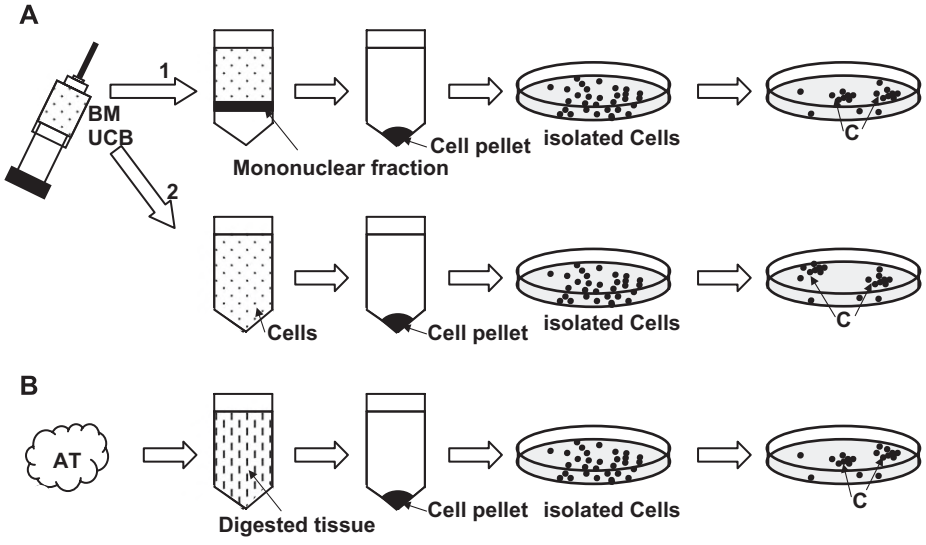

Figure 2. Isolation of mesenchymal stem cells from various tissues. Mesenchymal stem cells are routinely isolated and expanded from bone marrow (BM), umbilical cord blood (UCB) and adipose tissue (AT). A cell pellet containing the desired MSCs can be obtained via several isolation methods: (A1) through density-gradient centrifugation and plating of the mononuclear cell fraction, (A2) through plating the entire cell fraction after erythrolysis or cell sorting or (B) through plating the entire cell fraction after enzymatic digestion of the adipose tissue. These isolated cells are then plated at a high density in a culture dish. After a few days single cells adhere. These form colonies (C) and are the mesenchymal stem cells used for the experiments.

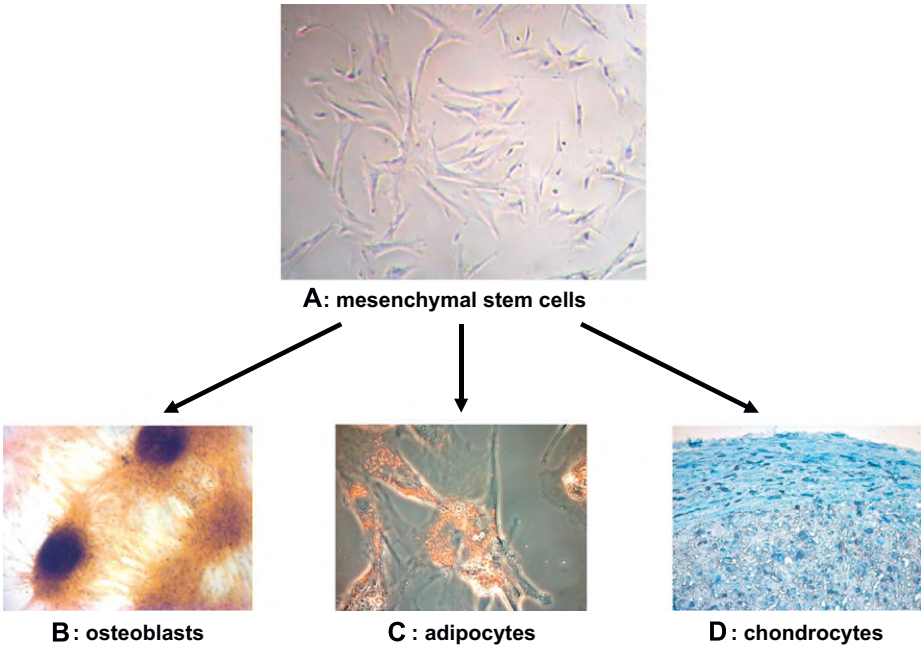

Figure 3. The multipotency of mesenchymal stem cells. Treatment with an osteogenic-specific induction medium causes the MSCs (A) to differentiate to osteoblasts (B) and deposit mineralized nodules that can be stained with von Kossa. After treatment with a specific adipogenic induction medium, MSCs contain a high amount of neutral lipids (stained with Oil red O), indicating their differentiation into adipocytes (C). In three-dimensional high-density culture, with an appropriate chondrogenic induction medium, MSCs differentiate to chondrocytes (D) and produce cartilage-specific proteoglycans (stained with alcian blue). magnification: (A and B) 200 $\times$; (C and D) 400 $\times$.

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 hematopoetic stem cell markers (Csaki et al., 2007; Dominici et al., 2006) (Figure 4). Parallel to bone-marrow-derived MSCs, MSCs are derived from umbilical cord blood. Umbilical cord blood has proven to be a good source of MSCs and umbilical-cord-blood-derived MSCs have been differentiated into multiple cell types such as endothelial cells, neurons, smooth muscle cells, adipocytes, chondroblasts and osteoblasts (Bieback et al., 2004; Kogler et al., 2004; Watt and Contreras, 2005; Aoki et al., 2004). Umbilical-cord-blood-derived MSCs have already become commercially available for horses and humans. Recently it has become increasingly fashionable to encourage parents to have their babies' umbilical cord blood deep frozen in case the need arises for later use. Also, the equine sporting industry has become a fore rider in collecting umbilical cord blood, hoping for regeneration of cartilage and tendon injuries with umbilical-cord-blood-derived MSCs (Koch et al., 2007). Apart from bone marrow and umbilical cord blood, adipose tissue also appears to be a good and plentiful source of MSCs both in humans and animals (Qu et al., 2007; Yamamoto et al., 2007; Zuk et al., 2001). Adipose-tissue-derived MSCs can be isolated from liposuctions in large numbers and after 1–2 h digestion of the adipose tissue with collagenase in a shaking water bath at 37 °C, easily grown under standard tissue culture conditions (Figure 2). The advantage over other methods of obtaining MSCs is that, for one, adipose tissue can be obtained by procedures that are minimal invasive and, MSCs yields obtained from adipose tissue are higher compared to other sources of MSCs such as bone marrow or umbilical cord blood (Helder et al., 2007; Kern et al., 2006). Although adipose-derived MSCs have been successfully differentiated *in vitro* into different lineage cells including adipogenic, chondrogenic, myogenic and osteogenic cells (Zuk et al., 2002), in the human, adipose-tissue-derived MSCs may have inferior chondropogenitor capacities compared to bone-marrow-derived MSCs (Im et al., 2005). However, in a comparative study of MSCs from bone marrow, adipose tissue and umbilical cord blood no phenotypic differences were observed (Wagner et al., 2005). There have also been reports of MSCs isolated from peripheral blood (Huss et al., 2000; Zvaifler et al., 2000; Ukai et al., 2007; Koerner et al., 2006; Giovannini et al., 2008), although yields are significantly lower and chondrogenic

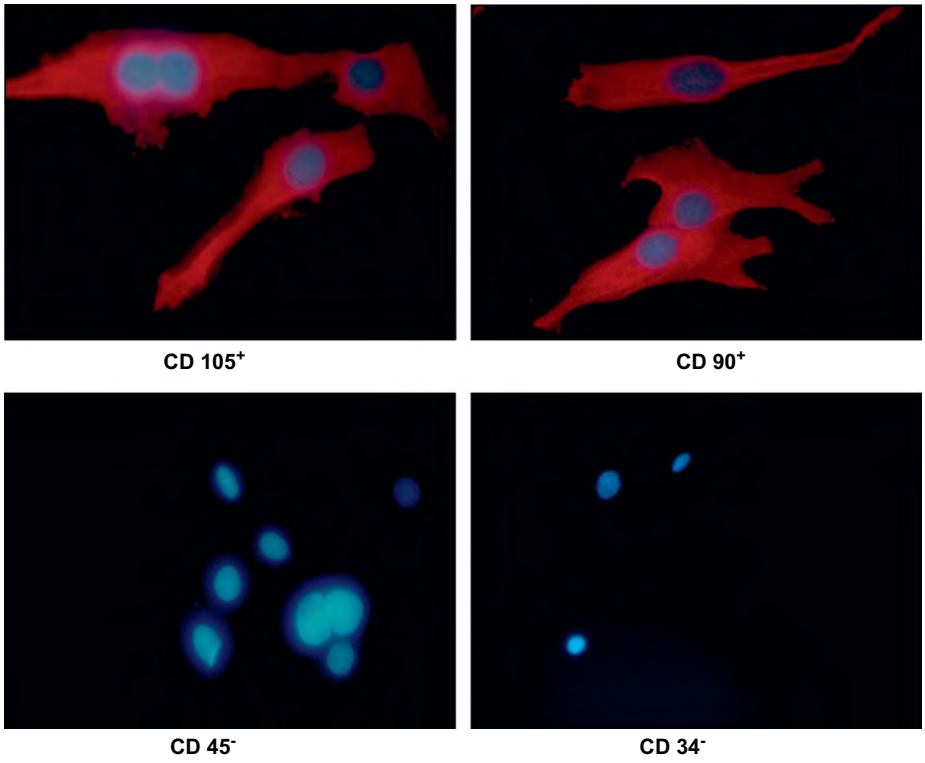

Figure 4. Immunolabeling of isolated mesenchymal stem cells. As defined by the International Society for Cellular Therapy, stem cells should exhibit the markers CD105 and CD90 and should not exhibit the hematopoetic stem cell markers CD45 and CD34. magnification: 200 $\times$.

induction is difficult to achieve. Recently, MSCs were even found to persist in adult cartilage (Alsalameh et al., 2004). Their role is still unclear and the authors of this study argue that fibrocartilage formation during OA might be a result of cytokine-induced wrong programming of cartilage MSCs (Alsalameh et al., 2004).

Induction of chondrogenesis in MSCs

In vivo MSCs differentiation is initiated through an interaction of molecular signals emitted from neighbouring tissue cells that are transduced via either an extra- or intracellular pathway. Differentiation of MSCs can then be induced through stimulation of stem cell surface receptors, soluble cytokine and growth factors, through ECM proteins such as proteoglycans and collagens or through direct interaction with surface proteins of neighbouring cells such as the chondrocytes. Present attempts on *in vitro* chondrogenic differentiation of MSCs are therefore based on the knowledge of chondrogenic development, cartilage homeostasis and function *in vivo*. Chondrogenic differentiation of MSCs *in vitro* is therefore mainly performed in a three-dimensional environment such as the micromass pellet culture as described by Johnstone et al. which is the most frequently used system (Pittenger et al., 1999; Johnstone et al., 1998). Here, 250,000–500,000 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 1 day in culture, the cells aggregate and form a round cell pellet. Other three-dimensional culture methods for cartilage formation include high-density bridge cultures (Figure 5) and alginate bead cultures (Lange et al., 2005; Shakibaei and De Souza, 1997; Shakibaei et al., 1993).

In vitro MSCs require a stimulus to differentiate into chondrocytes. This stimulus can be achieved with a large variety of different growth and differentiation factors, hormones or cytokines (Caplan and Goldberg, 1999; Magne et al., 2005). The major ones include TGF-β1, IGF-1, dexamethasone, the family of bone morphogenic proteins (BMPs) and fibroblast growth factor (FGF) (Carlberg et al., 2001; Csaki et al., 2007; Denker et al., 1999; Grigoriadis et al., 1988; Nakayama et al., 2003; Nixon et al., 2000; Pittenger et al., 1999; Tsutsumi et al., 2001).

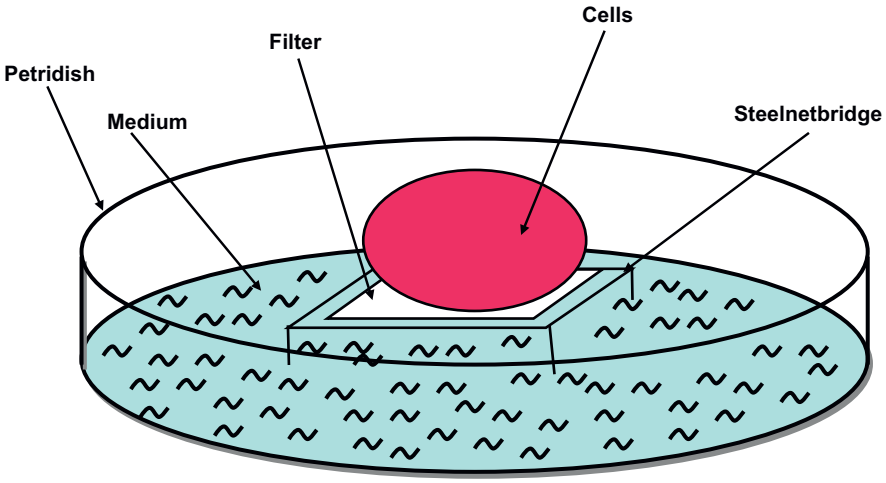

Figure 5. Schematic drawing of the three-dimensional High-Density Culture Model. A nitrocellulose filter is placed on a steel net bridge and cells are cultured on the filter. This model mimics the condensation phase that is at the beginning of cartilage development, by allowing the cells to aggregate and form cell–cell interactions. Cell-culture medium reaches the filter-medium interface, nurturing cells through diffusion, thus mimicking an *in vivo* environment.

In chondrogenic differentiation media, TGF-β1 is the most commonly used growth factor (Caplan, 1991; Pittenger et al., 1999; Johnstone et al., 1998). Chondrogenic differentiation through TGF-β1 is probably mediated by smad3 and β-catenin, as well as along the Wnt signaling pathway, enhancing MSCs proliferation while simultaneously inhibiting adipogenesis and osteogenesis (Zhou et al., 2004; Jian et al., 2006). Up-regulation of β-catenin is essential to commit a cell to the chondrogenic lineage (Day et al., 2005; Hill et al., 2005). However, in mature adult chondrocytes, β-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 enchondral bone growth plate. TGF-β1 has also been shown to act via the MAPKinase signaling pathway, part of its mechanism being mediated by ERK1/2; however, collagen type II production is independent of this (Longobardi et al., 2006). 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) and recently it was shown that ERK1/2 interacts physically with the chondrogenic transcription factor Sox9 (Shakibaei et al., 2006). This concurs with the findings that inhibition of the MAPKinase pathway enhances adipogenesis (Jaiswal et al., 2000). IGF-1 can influence chondrogenesis independently of TGF-β1 and may even be involved in a synergism with TGF-β1. Indeed, the expression of the chondrogenic-specific transcription factor Sox9, the amounts of collagen type II and cartilage-specific proteoglycans in MSCs stimulated both with TGF-β1 and IGF-1 were comparable to that of mature adult chondrocytes (Longobardi et al., 2006). Dexamethasone, a synthetic glucocorticoid, stimulates chondrogenesis directly via the glucocorticoid receptor (Derfoul et al., 2006). In animals it has been reported to be a potent stimulant for chondrogenesis in horses, rabbits and bovines (Bosnakovski et al., 2005; Grigoriadis et al., 1988; Johnstone et al., 1998). Interestingly, dexamethasone potentiates the chondrogenic stimulation of TGF-β (Derfoul et al., 2006). BMPs, belonging to the TGF-β superfamily can further stimulate chondrogenesis (Chubinskaya and Kuettner, 2003; Luyten et al., 1992; Sailor et al., 1996; Schmitt et al., 2003; Knippenberg et al., 2006; Indrawattana et al., 2004). Here especially BMP-7 (Knippenberg et al., 2006), a combination of BMP-2 and TGF-β (Schmitt et al., 2003) or a combination of BMP-6 and TGF-β (Indrawattana et al., 2004) seem to enhance chondrogenesis whereas BMP-2 alone stimulates osteogenesis (Knippenberg et al., 2006). BMP-2 seams to cooperate, like TGFβ, with the Wnt signal transduction pathway, up-regulating Wnt3a, leading to accumulation of β-catenin and the subsequent induction of Sox9 and chondrogenesis (Fischer et al., 2002a, b). FGF-2 has also been proposed to stimulate chondrogenesis in MSCs

(Solchaga et al., 2005a; Chiou et al., 2006) and this mechanism of action is mediated via the MAPKinase signaling pathway (Murakami et al., 2000).

Tissue-engineering cartilage with MSCs

Analogue to the ACT, MSCs have been injected in the knees of animals such as rabbit (Im et al., 2001; Yan and Yu, 2007) and goat (Quintavalla et al., 2002; Murphy et al., 2003). Although combining the MSCs with soluble scaffolds, fibrin glue or a periosteal flap, results have been ambiguous, both formation of new cartilage and degradation and fragmentation of the MSCs has been reported (Im et al., 2001; Yan and Yu, 2007; Murphy et al., 2003; Quintavalla et al., 2002). Further, MSCs have been implanted after *in vitro* differentiation to chondrocytes; however, also here results have been unsatisfactory (Jiang et al., 2003). The approach of loading MSCs onto a three-dimensional scaffold *in vitro* could provide a three-dimensional construct with mechanical properties that are congruent with the weight-bearing function of the joint (Noth et al., 2008). Therefore, MSCs have been implanted on scaffolds and indeed successful formation of cartilage-like tissue was observed in parts of the defect (Chen et al., 2005; Wakitani et al., 1994; Meinel et al., 2004; Liu et al., 2006). Attempts have been made such as press-coating MSCs onto the surface of a bio-degradable polymer or seeding the MSCs onto an amalgam scaffold of poly-L-lactic acid and alginate (Caterson et al., 2002, 2001). Recently, electrospun nanofiber scaffolds have come into focus of cartilage tissue engineering with MSCs (Li et al., 2002, 2005a, b; Yoshimoto et al., 2003).

Cartilage repair in clinical trials

To achieve clinical effectiveness, safety and practicality of using MSCs for cartilage repair will be necessary (Xian and Foster, 2006). As treatment of animals with MSCs has led to ambiguous results, clinical trials on human patients using MSCs for articular cartilage repair are scarce (Wakitani, 2007; Wakitani et al., 2002). Wakitani et al. (2002) performed a study on 24 human patients. In this study, autologous MSCs were obtained from the patients' bone marrow, expanded in monolayer culture, seeded onto a collagen type I membrane and transplanted into the cartilage defect. Twelve patients served as the control group and received cell-free implants. The 2-year outcome showed significantly greater hyaline cartilage formation in the treated compared to the untreated group. However, there was no way of tracking the

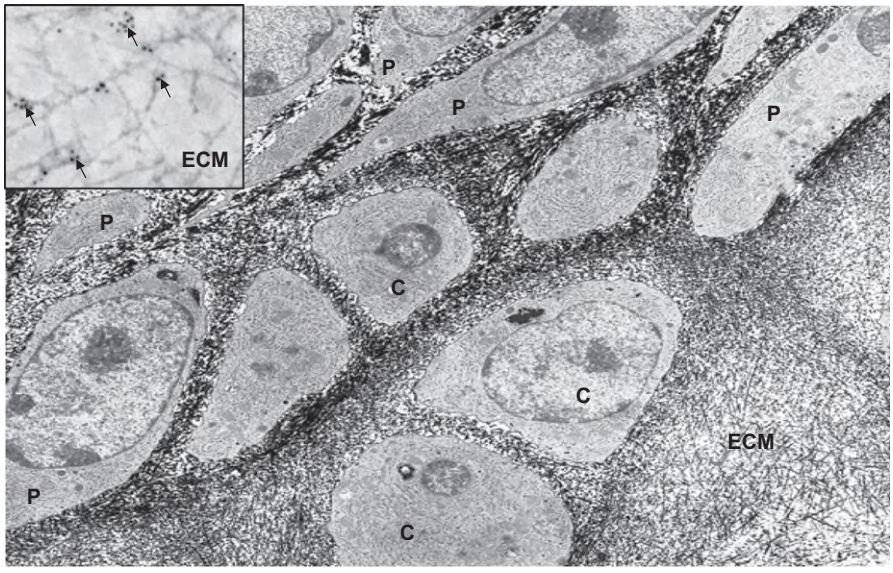

Figure 6. Transmission electron microscopy: High-Density Culture. Chondrogenic-induced MSCs differentiate into chondrocytes (C) and demonstrate typical cartilage nodule formation with deposition of cartilage-specific extracellular matrix (ECM) in high-density culture conditions. Cartilage nodules are surrounded by a layer of flattened fibroblast-like MSCs, resembling a perichondrium-like structure (P). During appositional growth of the cartilage nodule, MSCs are recruited from this outer layer. This transitional zone is shown in the picture: MSCs with a flattened fibroblast morphology and large, thick fibrillar ECM production differentiate into smaller, rounded chondrocytes and produce fine-structured ECM. Magnification: 6500 × ; Inset: immunolabeling demonstration of collagen type II after 7 days in culture. Collagen fibrils are present and gold particles are detectable only on collagenous fibrils in the cartilage matrix (arrows). Magnification: 75,000 × .

implanted MSCs for this long time period, so it remains unclear whether the newly formed tissue consisted of the implanted MSCs.

Hurdles in tissue-engineering cartilage from MSCs

A major hurdle in cartilage tissue engineering with MSCs is inadequate aging of the tissue-engineered constructs. During *in vitro* chondrogenesis, MSCs not only up-regulate hyaline cartilage-specific markers such as collagen type II (Figure 6) and adequate cartilage-specific proteins such as aggrecan, but also markers typical for hypertrophic chondrocytes such as collagen type X and alkaline phosphatase (Johnstone et al., 1998). Collagen type X makes up 45% of the collagen produced in hypertrophic chondrocytes and is therefore considered an important marker of enchondral bone formation (Shen, 2005; Gibson and Flint, 1985). In chondrogenic differentiated MSCs, collagen type X is considerably up-regulated in three-dimensional culture and detectable around day 7 with RT-PCR (Johnstone et al., 1998; Barry et al., 2001) and around day 14 with immunohistochemistry (Nishioka et al., 2005; Yoo et al., 1998). In contrast, in healthy mature chondrocytes and in engineered cartilage from mature chondrocytes, collagen type X is not or is only marginally expressed (Zhang et al., 2004; Riesle et al., 1998; Tallheden et al., 2004).

Alkaline phosphatase activity, generally regarded as a typical marker for osteogenesis can also be found in large amounts in hypertrophic chondrocytes in the calcified zone, in enchondral ossification centers and the growth plate (Henson et al., 1995; Miao and Scutt, 2002). During chondrogenic induction, MSCs upregulate alkaline phosphatase production around the 7th day, reaching a peak around the 14th day (Johnstone et al., 1998). In contrast to MSCs, in adult mature chondrocytes from the superficial and the middle zone of the joint surface, Alkaline phosphatase activity is minimal (Henson et al., 1995; Miao and Scutt, 2002).

Conclusion

In conclusion, MSCs present themselves as promising, attractive tools for cartilage repair. Their properties make them ideal to study the development, physiology and disease of cartilage. MSCs can be obtained rather easily from a great number of tissues of which the most suitable seem to be bone marrow, umbilical cord blood and adipose tissue. One of their great advantages, in contrast to chondrocytes, is that they have a high proliferative potential in culture and can be expanded to obtain enough cells for tissue engineering of cartilage. Today, tissue engineering and stem cell technologies have established themselves as approved new approaches especially in cartilage and OA research. However, although research with MSCs for cartilage and connective tissue repair has come a long way, we are still only at the beginning of this exciting new journey. Future studies will need to investigate mechanisms of MSC differentiation and the biochemical signal transduction pathways involved in maintaining and enhancing chondrogenic differentiation in even more detail. Understanding the biology of MSCs and their interaction with three-dimensional scaffolds will enable us to create more appropriate biomaterials capable of replacing cartilage defects and eventually make our dream of forming ''laboratory-made'' connective tissues including cartilage, bone and tendon come true.

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