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Joint Cartilage Tissue Engineering and Pre-Clinical Safety and Efficacy Testing

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1. Introduction

1.1 Cartilage injuries, the triad and quartet of tissue engineering

Presently, focal cartilage injuries in humans are treated by abrasion arthroplasty with or without subchondral bone microfracture, autologous chondrocyte or mesenchymal stem cells (MSC) implantation and osteochondral plugs used in a sequential fashion pending severity and duration of the problem (Figure 1) (Williams et al., 2010; Steinert et al., 2007). Current strategies in human medicine for treatment of diffuse joint degeneration rely on replacement of the whole degenerated joint with inert implants. Excellent treatment outcome has been achieved for up to 15 years or more, but approximately 20% of treated patients require revision procedures after this time (Steadman et al., 2001). For younger patients this current state-of-the-art may translate to two or more revision surgeries during their lifetime. A biological solution to repair damaged cartilage that would provide life-long pain relief would be a major medical achievement.

The tissue engineering triad refers to the use of cells, scaffolds and cytokines to engineer tissues in vitro or in vivo. Such engineered tissues can potentially be utilized for tissue replacement strategies, for pharmacological screening of agents for therapeutic or toxic effects, or to gain insight into tissue developmental processes. Cartilage tissue, engineered using this triad of components often exhibit hyaline cartilage morphology, but the tissue has inferior mechanical properties when compared to native joint cartilage (Grad et al., 2011; Schulz and Bader, 2007). A fourth component of tissue engineering, namely mechanical stimulation has been added to the classical triad in order to better replicate the in vivo environment of joint cartilage (Grad et al., 2011; Schulz and Bader, 2007). Using this "quartet" of tissue engineering (cells, scaffolds, cytokines, and mechanical stimuli), cartilage

with better mechanical properties has been produced. The mechanical properties of cartilage produced using mechanical stimulation may or may not be better than static culture depending on the cell source, timing of mechanical stimulation, method and duration of mechanical stimulation, and other variables. It is clear that each component of this tissue engineering "quartet" should be studied in detail since different cells respond differently to different cytokines, scaffold composition and topography as well as mode and timing of mechanical stimuli. This underlines the complexity of tissue engineering where each of these areas is a separate research field.

Here, we will briefly discuss the cells used for cartilage tissue engineering followed by more detailed discussion of selected topics on scaffolds, cytokines and means of mechanical stimuli. Pre-clinical animal models of cartilage repair and future perspectives follow these tissue-engineering considerations.

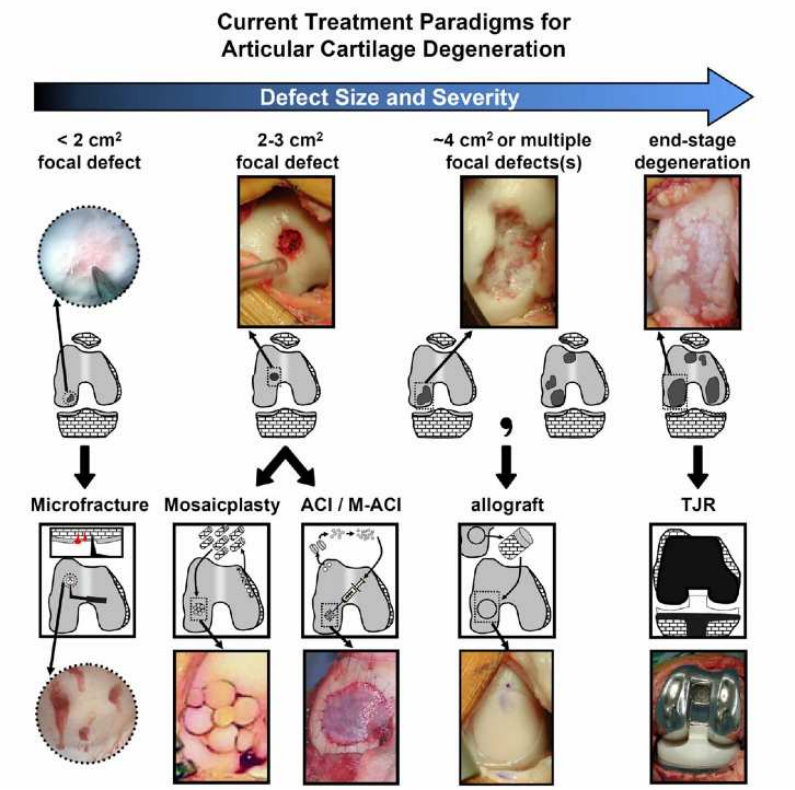

Fig. 1. Current articular cartilage treatment algorithm. ACI = autologous chondrocyte implantation, MACI = matrix-assisted autologous chondrocyte implantation, TJR = total joint replacement. Reprinted with permission from Williams et al. (2010)

2. Cells of cartilage tissue engineering

Articular hyaline cartilage is a very specialized tissue characterized by low cellularity, extensive extracellular matrix, lack of vascular, lymphatic, and nervous supply, and

insufficient number of resident progenitor cells needed for mounting an appropriate regenerative response to injury. The result is minimal intrinsic repair capacity.
The obvious choice of cell for cartilage repair is the chondrocyte from hyaline cartilage since it is normally responsible for the production and maintenance of joint cartilage. However, a number of factors have limited the use or therapeutic success of chondrocytes. Chondrocytes only constitute about 5% of joint cartilage and insufficient chondrocyte numbers are therefore often retrieved for immediate therapeutic use, which in turn necessitate ex vivo cell expansion prior to re-implantation. Chondrocytes unfortunately tend to dedifferentiation towards the fibroblast cell lineage when expanded in culture making them less suitable for transplantation (Schulze-Tanzil, 2009). Another limitation to the use of autologous chondrocytes is the need for two surgical procedures, weeks apart to harvest and later implant the cells, which adds time, cost, anesthetic risk and risks of donor site morbidity (Schulze-Tanzil, 2009; Williams et al., 2010). Interestingly, osteoarthritic chondrocytes seem to perform equally well in vitro when compared to chondrocytes from healthy joints (Dehne et al., 2009; Stoop et al., 2007).
Stem cells or cells with chondrogenic potential from various tissue sources have been investigated for cartilage repair. Adult cells such as bone marrow multipotent stromal cells (BM-MSCs) are attractive due to the decreased cost and risks associated with collection compared to autologous chondrocytes. Concerns raised with the use of BM-MSCs include potentially a decreased yield and differentiation potential of MSCs from the bone marrow with increasing age making the technique age-dependent. This is paradoxical to the fact that clinical cases often occur in aged individuals (Wilson et al., 2010; Kasper et al., 2009). Adult cells with chondrogenic potential have been isolated from a large number of tissues and have been reviewed elsewhere (Solchaga et al., 2011; Hildner et al., 2011; O'Sullivan et al., 2011). Embryonic and recently, induced pluripotent stem cells (iPS cells), may also be valuable cell sources for cartilage repair (Hiramatsu et al., 2011; Medvedev et al., 2010; Waese and Stanford, 2011; Fecek et al., 2008). Neonatal stem cells with chondrogenic potential have been isolated from fresh umbilical cord blood of humans as well as horses (Berg et al., 2009; Koch et al., 2007; Santourlidis et al., 2011; Zhang et al., 2011). Human cord blood derived stem cells have showed increased proliferation capacity and broader differentiation potential compared to stem cells from bone marrow and adipose tissue (Zhang et al., 2011; Kogler et al., 2004). Also, cord blood MSCs may be more chondrogenic than bone marrow MSCs (Berg et al., 2009; Zhang et al., 2011).
So far a reliable cell source and method of isolating cells with high chondrogenic potential has not been reported. From a tissue-engineering point of view this is a major limitation at the moment, since it precludes development of predictable and reproducible protocols for cartilage production.

3. Biomaterials & scaffold fabrication technologies

Joint cartilage regeneration can be achieved by two strategies, namely cell- and scaffold-based therapies. Cell therapy has already reached the clinics in the form of autologous chondrocyte implantation (ACI) or matrix-assisted ACI (MACI). However, this regenerating technique appears to assist only partially in the repair process as it leads to the formation of mechanically inferior fibrocartilage compared to native joint cartilage. Although clinical outcome is better five years after surgery when compared to the baseline, recent comparative studies showed that ACI and MACI were not significantly better than marrow-stimulating techniques (e.g. microfractures) or reconstructive techniques (e.g.

mosaicarthroplasty) using autografts, allografts or synthetic material (Ebert et al., 2011; Vasiliadis et al., 2010). The influence of scaffold composition and surface topography on cell function and differentiation is being increasingly recognized (Rosso et al., 2005; Milner and Siedlecki, 2007; Thakar et al., 2008; Patel et al., 2010). Improvement to the MACI technique and other scaffold-based approaches may therefore be made through precise engineering of three-dimensional (3D) porous biomaterials – 3D scaffolds – that promote cell lodging, migration, and differentiation while providing mechanical support during tissue repair.
Different biomaterials are used to fabricate scaffolds, predominantly natural and synthetic polymers. In general two classes of polymers can be distinguished: (i) hydrogels formulated as cell carriers for minimally invasive surgeries; (ii) solid polymers designed for optimal mechanical stability. Hereafter, these biomaterials, their physicochemical and mechanical properties, and the correspondent fabrication technologies implemented to make 3D scaffolds will be discussed.

3.1 Hydrogels

A number of general properties should be possessed by hydrogels. Obviously, these materials need to elicit an appropriate host response and display satisfactory biocompatibility. If a specific hydrogel composition would be associated with prolonged inflammation, the resulting immune response toward the encapsulated cells might affect the success of the implantation. The gelation mechanism is also important. Typically hydrogels are formed by ionic or covalent cross-linking. Ionic cross-links are very dynamic and may be formed and disrupted in presence of a multivalent ionic fluid environment, which is like physiological fluids. This may hamper the control over the degradation properties of the resulting hydrogels. Conversely, covalent cross-links are more stable and confer enhanced mechanical and physical properties to the hydrogels. Yet, they are often more toxic and more difficult to break than ionic cross-links. This implies that the cross-linking yield should always be maximum and different routes for hydrogel degradation should be envisioned in the design of covalent cross-linking biodegradable hydrogels (Lee and Mooney, 2001). Alternative methods to form cross-links have been developed by exploiting the phase transition characteristics of polymers. Temperature sensitive hydrogels have been synthesized for tissue engineering applications. These polymers are subjected to a solid to gel phase transition at a specific temperature, called lower critical solution temperature, which can be tailored to be at body temperature (Fedorovich et al., 2009; Vihola et al., 2005). Alternatively, stereocomplexation of amphiphilic polymers has also been used as a strategy to form physical cross-linked hydrogels. These hydrogels display customized physical and mechanical properties depending on the polymer molecular weight and relative concentration of the amphiphilic blocks (Hiemstra et al., 2005; Hiemstra et al., 2006b). Finally, mechanical and degradation properties are important to consider when choosing a hydrogel for a specific tissue. Ideally, a hydrogel should possess a similar stiffness to that of the targeted tissue and a degradation rate matching the speed of tissue formation.
Natural hydrogels. Among natural hydrogels, alginate, chitosan, hyaluronic acid and its derivatives, and collagen have been widely investigated for cartilage repair. Alginate is derived from brown algae and is composed of linear block copolymers of mannuronic (M) and guluronic (G) acids. It has been widely used in drug delivery and tissue engineering applications because of its biocompatibility, relative low cost, abundance, and easy gelation in presence of divalent cations (Shapiro and Cohen, 1997; Terada et al., 2005). Ionic cross-

links are very dynamic and can lead to unpredictable and uncontrollable dissolution of the gel in a physiological environment. Therefore covalent cross-links were introduced and were shown to improve the mechanical properties and the control over the degradation rate of these hydrogels. Alginate is a rather inert biomaterial. In order to enhance cell-material interactions, alginate hydrogels have been modified with different biological factors to promote cell adhesion or differentiation into specific tissue phenotypes (Hao et al., 2007; Hsiong et al., 2008). An important issue is that alginate degradation products often are larger than the threshold size for renal clearance. Although previous approaches revealed new opportunities to control the degradation of alginates (Lee et al., 2000), more efforts are needed to translate these gels into the clinics.

Chitosan is a polysaccharide forming the exoskeleton of many seashells. As it is mainly comprised of glucosamine, a component of cartilage extra-cellular matrix (ECM), chitosan is an attractive biomaterial for regenerative therapies of the skeletal system (Chenite et al., 2000; Madihally and Matthew, 1999). Its positive charge allows complexation with other negatively charged ECM proteins present in articular cartilage like glycosaminoglycans. This would induce *in vivo* sequestration of growth factors embedded in the surrounding cartilaginous ECM. Chitosan can be processed in the form of a gel exploiting its pH-dependent solubility, drawn into solid fibers, or foamed by freeze-drying (Chenite et al., 2000; Yamane et al., 2005). This processing versatility enables the fabrication of scaffolds with fine-tunable mechanical properties. Although the intrinsic batch-to-batch variability during chitosan extraction is still a major drawback, its physical, mechanical, and biological properties are promising for articular cartilage regeneration.

Hyaluronic acid is a highly hydrophilic proteoglycan present in the extra-cellular matrix (ECM) of several tissues. In cartilage, it contributes to maintain homeostasis and physical integrity thanks to its viscosity, capacity to retain water, and interactions with chondrocytes and other ECM proteins. When used as a biomaterial, hyaluronic acid can be covalently cross-linked or esterified to improve its mechanical properties and physical stability. In the latter case, hylans are formed. These biomaterials are currently used in the clinics as hydrogels or membranes to assist in autologous chondrocyte implantation (Brun et al., 1999; Campoccia et al., 1998; Hollander et al., 2006). Yet, the less-than-optimal mechanical properties and the potential presence of endotoxins and impurities are drawbacks that still need to be improved to consider these biomaterials a fully viable alternative to synthetic polymers. Furthermore, hyaluronic acid is present in high concentrations in the surrounding tissues of malignant tumors (Knudson et al., 1989). This evidence should be further studied to ensure that no risks are associated with the use of hyaluronic acids or its derivatives as biomaterials.

Collagen is one of the extra cellular matrix protein most used to form natural hydrogels. It is easy to obtain from different sources as it is the most common protein present in numerous tissues and organs. Jellification typically occurs through thermally reversible physical cross-links. These gels can be easily remodeled through production of collagenases by encapsulated cells, thus offering the opportunity to study cell-matrix interactions during tissue development (Mueller et al., 1999; Pachence, 1996; Nehrer et al., 1997). Collagen gels can also be formed in the presence of other proteins such as chondroitin sulphate or hyaluronic acid, so that the resulting semi-interpenetrating network can be functionalized to display biological moieties of interest in cartilage regeneration. Gelatin, a derivative of collagen formed by breaking its natural triple-helix structure, has also been used as a

hydrogel for cartilage regeneration (Choi et al., 1999). The main drawbacks of collagen and its derivatives lye in the potential immunogenic risks due to its origin, batch variation, and poor mechanical properties. Chemical cross-linking strategies by exposure to glutaraldehyde or genipin confer enhanced physical stability and mechanical properties to the resulting hydrogels, however without reaching similar stiffness to that of articular cartilage.

Synthetic hydrogels. Among synthetic hydrogels, a number of derivatives based on poly(ethylene glycol) (PEG) have been developed and used for cartilage tissue engineering. This biomaterial is relatively inert, highly hydrophilic, and very versatile for the functionalization of its backbone with different biological moieties. PEG is known to have anti-fouling properties and is often used in vascular applications as a coating to prevent thrombogenesis. PEG can be synthesized either as a linear, branched, or star-shaped polymer. In its star-shaped form, different peptides or growth factors can be coupled depending on the number of arms. These biological factors can be covalently linked to the PEG chains through passive or active linkers comprised of an enzymatically sensitive peptide sequence, thus being able to release the payload depending on cellular activity (Adelow et al., 2008; Lutolf et al., 2003). PEG or its oxidized version poly ethylene oxide (PEO) can also be grafted to other polymers such as poly propylene oxide (PPO) or poly lactic acid (PLA). PEO-PPO-PEO is a tri-block copolymer, also commercially known as pluronic®, that jellifies through a temperature sensitive phase change. These copolymers have been conventionally used as drug delivery vehicles and recently also explored in tissue engineering applications for skeletal regeneration (Fedorovich et al., 2009; Park et al., 2009; Batrakova and Kabanov, 2008). Yet, PEO-PPO copolymers are not degradable limiting their potential use. PEG-PLA copolymers have been synthesized for tissue engineering applications. Gelation occurs via stereocomplexation of L- and D-lactic acid. By changing the molecular weight of the PLA and PEG blocks, it is possible to vary the mechanical properties of the resulting gels. Proteins and other biological factors can be easily incorporated during gelation in these degradable hydrogels, conferring them a high versatility and potential for clinical applications (Hiemstra et al., 2006a; Hiemstra et al., 2006c). An alternative derivative of PEG is synthesized by linking acrylate groups to the main chain. PEG-diacrylate (PEGDA) is a photopolymerizable hydrogel that cross-links in presence of an initiator under UV light. PEGDA has been extensively used in cartilage tissue engineering as an inert encapsulating system or after modification with different peptides, growth factors, or in semi-interpenetrating networks with ECM proteins (Hwang et al., 2006; Lee et al., 2006; Sharma et al., 2007). It is a reproducible hydrogel system with fine-tunable physicochemical and mechanical properties that enable tissue regeneration without the potential risks associated to natural polymers, thus making it a promising candidate for clinical use.

3.2 Solid polymers

Within solid polymers, poly lactic acid (PLA), poly glycolic acid (PGA) and copolymers (PLGA) have been broadly used in tissue engineering as well as for cartilage regeneration (Anderson and Langone, 1999; Babensee et al., 2000; Chu et al., 1997; Freed et al., 1993; Honda et al., 2000; Sarazin et al., 2004). These biomaterials are approved by the food and drug administration (FDA) as they activate a minimal or mild foreign body reaction, and as such are considered biocompatible. The mechanical properties and degradation rate can be

tailored by varying the molecular weight and copolymer ratio. They have already been studied for drug delivery (Jang and Shea, 2003; Uhrich et al., 1999; Richardson et al., 2001; Nof and Shea, 2002; Sengupta et al., 2005) and are suitable for tissue engineering applications, as the degradation products (lactic and glycolic acids) obtained due to hydrolysis are normally present in the metabolic pathway and can be naturally eliminated by the body. However, their bulk degradation may be associated with the formation and accumulation of large amounts of degradation products in a short time frame (months vs. years) that cannot be easily discarded, resulting in local inflammation in tissues (Bostman et al., 1989) and enzymatic hydrolysis (Fu et al., 2000). Another polyester commonly used in tissue engineering is poly ε-caprolactone (PCL). This polymer is characterized by good biocompatibility and mechanical properties. It degrades at a much slower rate than PLA, PGA, and PLGA, which makes it attractive when long-term implants and controlled release applications are desired (Honda et al., 2000; Wang, 1989; Hutmacher et al., 2001; Choi and Park, 2002). It has been also shown that PCL can selectively adsorb vitronectin, a protein that is known to facilitate stem cell chondrogenic differentiation. Conversely, PLA selectively adsorb fibronectin and seems to be better suited to induce stem cell osteogenic differentiation. A different family of thermoplastic polymers that has been investigated for tissue engineering is poly ethylene oxide terephthalate-co-poly butylene terephtalate (PEOT/PBT). These polyether-ester copolymers possess good physical properties like elasticity, toughness and strength (Bezemer et al., 1999). By varying the molecular weight of the starting PEG segments and the weight ratio of PEOT and PBT blocks, it is possible to tailor physico-chemical and mechanical properties (Bezemer et al., 1999; van Dijkhuizen-Radersma et al., 2002; Deschamps et al., 2002; Olde Riekerink et al., 2003; Woodfield et al., 2004; Moroni et al., 2006b), degradation rate (Deschamps et al., 2002), and protein adsorption (Mahmood et al., 2004). PEOT/PBT copolymers have been demonstrated to be biocompatible both in vitro and in vivo for skin, cartilage, and bone regeneration (Malda et al., 2004; van Blitterswijk et al., 1993; Bakker et al., 1988; Beumer et al., 1994a; Beumer et al., 1994b). A further modulation in degradation rate and drug release profile can be achieved by substituting part or all of the terephtalate domains with succinate blocks during the copolymerization reaction (van Dijkhuizen-Radersma et al., 2003; van Dijkhuizen-Radersma et al., 2004; van Dijkhuizen-Radersma et al., 2005). PLA, PGA, PLGA copolymers, PCL, and PEOT/PBT copolymers have proven to be interesting biomaterials to fabricate 3D scaffolds. Although their properties can be customized for specific purposes, some concerns over their degradation mechanism and rate still remain.

To obviate bulk degradation, surface eroding polymers have been developed such as polyortho-esters (POEs) (Choi and Heller, 1978), polyphospho-esters (PPEs) (Wang et al., 2001a; Wang et al., 2001b), and polyanhydrides (PAs) (Leong et al., 1986). Surface erosion is a degradation mechanism (Andriano et al., 1999; Burkoth et al., 2000), which affects the stability of the scaffolds to a lesser extent and elicits a lower in vivo inflammatory response, as compared to polyesters and polyether-esters previously considered. PPEs display adequate mechanical properties also for hard tissue engineering. Although PPAs, PAs, and POEs have been used in some cases for hard tissues repair, they might be more suitable for soft tissue engineering due to their generally low mechanical properties. More recently, PLA based polymers modified with photosensible chemical groups like fumarates or acrylates have been developed for biomedical applications (Melchels et al., 2009; Melchels et al., 2006). These biomaterials offer the advantage of being processed by rapid prototyping technologies with high resolution, thus enabling the fabrication of

sophisticated scaffold geometries. Yet, some issues may still arise from remnant toxicity due to the acrylic groups.

3.3 Scaffold fabrication technologies

A plethora of fabrication technologies have been developed and characterized to fabricate three-dimensional (3D) scaffolds for tissue engineering applications (Figure 2). Although these techniques can also be applied to hydrogels, they have been mostly used with solid polymers due to the intrinsic advantage of the former as a minimally invasive injectable biomaterial. Foams and textiles are the two predominant types of scaffolds used in tissue engineering. Foams can be fabricated by gas foaming, freeze drying, or porogen leaching (Barry et al., 2004; Sproule et al., 2004; Schoof et al., 2001; Ma and Zhang, 2001; Claase et al., 2003; Sarazin and Favis, 2003). Textile scaffolds can be produced by wet or melt spinning, creating fibers that are randomly deposited on top of each other, woven, or knitted (Cima et al., 1991; Freed et al., 1994; Niklason and Langer, 1997). Once deposited, thermal or chemical treatments can be applied to improve fiber bonding, thus enhancing structural stability and mechanical properties (Kim and Mooney, 1998a; Kim and Mooney, 1998b; Mikos et al., 1993). These methods are relatively easy to implement, but offer a limited control over mechanical properties, interconnectivity of of pore network, pore size and shape, and porosity. It has recently been shown that scaffolds mimicking the structural and architectural characteristics of the targeted tissue support enhanced tissue formation, and fabrication technologies that enable a fine control over scaffold pore network and strut size are needed. In the specific case of articular cartilage, three different zones can be distinguished: (i) deep, (ii) middle, and (iii) surface zones. In each of these regions, the alignment of collagen type II is distinct and results in the overall arch-like architecture typical of articular cartilage. Furthermore, chondrocytes behave in a different manner and are responsible for the production of different extra-cellular matrix proteins, namely different proteoglycans, depending on the zone where they are located (Klein et al., 2009; Klein et al., 2003; Schuurman et al., 2009). This has lead researchers to focus their attention on technologies that enable the fabrication of layer-by-layer scaffolds. This would enable not only a better control of different cartilage zones, but also the construction of more complex tissue such as osteochondral grafts. A simple and effective solution is to cross-link monolithic hydrogel regions containing chondrocytes of the different regions or other cells on top of each other (Elisseeff et al., 2002; Lee et al., 2008; Hwang et al., 2007).

Where a better control over cell and tissue spatial distribution is desired, rapid prototyping (RP) technologies offer an appealing solution. RP is based on computer aided design (CAD) and computer aided manufacturing (CAM) to build porous 3D scaffolds in a layer-by-layer controlled manner. An extensive number of biomaterials can be processed by these techniques in a custom-made shape (Hutmacher, 2001; Yang et al., 2002; Yeong et al., 2004), with tailored mechanical properties to the specific application considered (Hollister, 2005; Taboas et al., 2003; Lin et al., 2004). The outcomes are 3D scaffolds that possess fine-tunable porosity, pore size and shape, and have a completely interconnected pore network, which permits a better cell migration and nutrient perfusion than 3D scaffolds fabricated with more conventional techniques like foaming or spinning (Malda et al., 2004; Sachlos and Czernuszka, 2003). In addition, the fabrication of personalized scaffolds can be envisioned with the acquisition and processing of computer tomography (CT) and/or magnetic resonant imaging (MRI) anatomical data from patient datasets (Hollister, 2005; Moroni et al.,

2007a). Since RP is based on layer-by-layer processing, it is also theoretically possible to change the pore network structural and architectural characteristics in space in order to better mimic specific ECM and cell spatial arrangement. In practice, this has not been extensively explored. Moroni et al. (Moroni et al., 2006a) have been active in studying how mechanical properties can be optimized with different pore networks for the regeneration of different types of cartilage. Similarly, Woodfield et al. (2005) produced scaffolds with pores varying along the longitudinal axis in the attempt to mimic the spatial distribution of chondrocytes in articular cartilage. Oh et al. (2010) used a similar approach and fabricated scaffolds with different pore size ranges by a more conventional freeze-drying method. In these scaffolds, chondrogenic differentiation in adipose derived mesenchymal stem cells was better supported with pores in the 370-400 μm range. The resulting scaffolds showed enhanced tissue formation, while cell and ECM distribution resembled more closely that of native hyaline cartilage. By combining different materials, it was also possible to fabricate osteochondral constructs that functionally supported both bone and cartilage regeneration (Moroni et al., 2008; Sherwood et al., 2002).

From these studies it is clear that 3D scaffolds fabricated by RP offer many advantages and a broad flexibility as a model to study new strategies for tissue engineering. However, they do not possess biologically active properties to improve communication with the adhered or encapsulated cells. This can be achieved by two means: (i) implementing drug delivery vehicles into the scaffolds, (ii) improving the fabrication resolution to achieve true synthetic ECM substitutes. In the former case, biological factors have been encapsulated or covalently linked into hydrogels (Ehrbar et al., 2007; Saha et al., 2007; Kong and Mooney, 2007) or incorporated into microspheres and fibers (Martins et al., 2010; Richardson et al., 2001), which were directly used for tissue engineering applications (see Delivery Methods section). Controlled drug delivery strategies showed to improve cartilage regeneration when combined with 3D scaffolds. However, local and spatial control over the release of biological factor is still lacking and might contribute to better mimic the native tissue architecture during regeneration. In the latter case, 3D scaffolds with feature dimensions in the range of ECM have been recently fabricated by two-photon polymerization (2PP). This technology exploits *in situ* polymerization of photosensible polymers at specific wavelengths (Ovsianikov et al., 2011). With 2PP, a number of rather complex structures can be fabricated with nanometric resolution (Ovsianikov et al., 2010). These can be interesting tools to study fundamental cell-material interaction at a single cell level, but the fabrication time window might be too long when clinically relevant scaffold dimensions are needed. An alternative technique that has been extensively used to mimic the ECM of tissues is electrospinning (ESP). Here, fiber meshes are fabricated by spinning a biomaterial solution into an electric field that destabilizes the solution flow and form a continuous jet of fibers collected on a target plate. The fiber deposition architecture can be modified depending on the geometry and electric properties of the collector plate (Zhang and Chang, 2008; Zhang and Chang, 2007). ESP has been used with a large number of biomaterials (Li and Xia, 2004) and also enables the incorporation of growth factors, proteins, or cells during fabrication (Li et al., 2006a; Patel et al., 2008). The fabricated scaffolds possess physical and surface properties that have already been shown to support cartilage tissue engineering (Li et al., 2006b; Li et al., 2005). These meshes may suffer from a lack of cell penetration due to the high fiber density and small pore size. However, spinning of more biomaterials with different degradation rate can obviate this. Virtually, water-soluble biomaterials would allow a fast degradation and a better infiltration of cells into the scaffold pores.

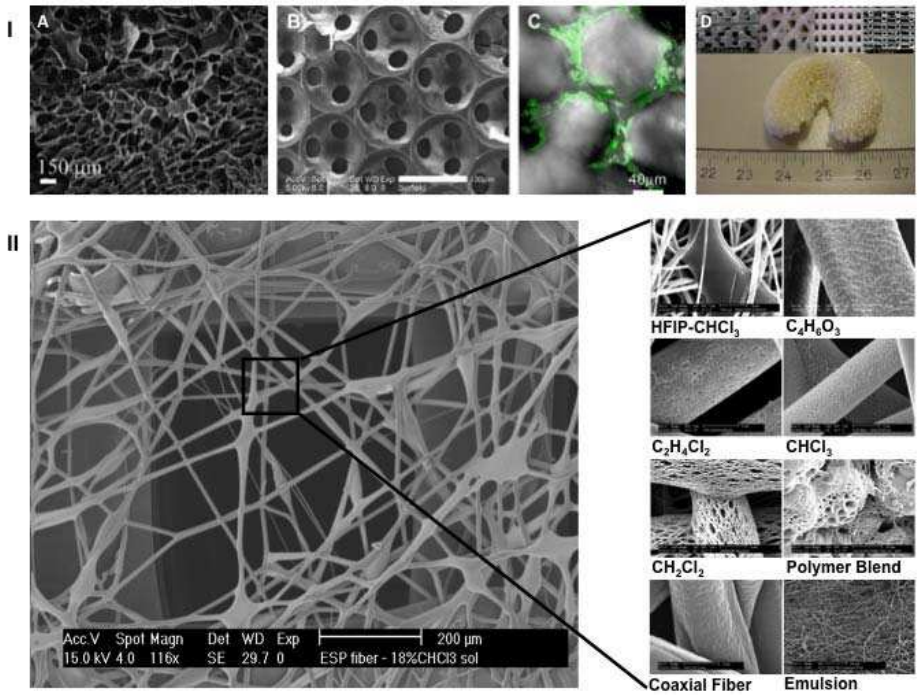

Fig. 2. Scaffold fabrication technologies. Different scaffold fabrication technologies can be used to create 3D porous biomaterials for tissue engineering applications. (I) Conventional foams can be obtained by (I-A) salt leaching or (I-B, I-C) by inverted colloidal crystal (ICC) template. In both case a porogen material is used to form a defined volume with the selected biomaterial and later removed through selective dissolution. In case of ICC, the resulting pore network is improved in terms of interconnectivity and (C) cell seeding efficiency (cells green fluorescent). (I-D) Textile scaffolds can be fabricated by rapid prototyping technologies. (I-D) Here, a meniscus shaped scaffold is shown. Insets display different fiber deposition methods, which affect the formed pore size and shapes. Not only solid polymers, but also hydrogels can be processed (no picture shown but available at Landers et al (2002) (Landers et al., 2002). (II) ECM mimicking meshes can be fabricated by electrospinning. (III-A) The electrospun fibers typically have a smooth surface, but (II-B) depending on the solvent used can also display different surface morphologies. Panel (I-A, I-B, I-C) modified by Deschamps et al (2002) and Nichols et al. (2009). Panel (I-D) modified by Moroni et al (2007). Panel (II) modified by Moroni et al. (2008)

4. Cytokines release

Cartilage engineering is not only a result of cells and scaffolds coming together to form a 'cartilage-like' structure. As mentioned earlier there are two relevant cell choices – chondrocytes and MSCs. Chondrocytes need to be in an environment resembling the physiological properties of cartilage to maintain their phenotype. MSCs will need to go

through the process of chondrogenesis. Chondrogenesis is an intricate process where MSCs go through stages of differentiation to become fully matured chondrocytes (Figure 3). Only when the cells have committed to the chondrogenic fate will they start to lay down cartilage-type extracellular matrix (ECM). The differentiation process involves a range of stage specific molecules that include phenotypic determinants, adhesion molecules, and signalling molecules as described in great detail by Chen et al. (2009).

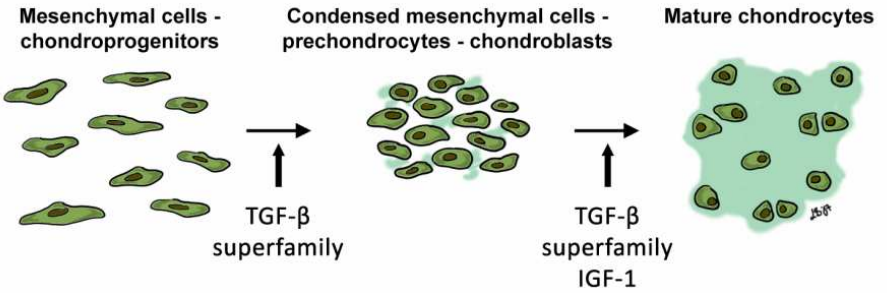

Fig. 3. Chondrogenic differentiation. Chondrogenesis is an intricate process where MSCs go through stages of differentiation to become fully matured chondrocytes. Members of the TGF-β superfamily and IGF-1 are examples of important regulators in the differentiation process. Figure by L. C. Berg

When attempting to produce tissue that resembles true cartilage both chondrocytes and MSCs will need the right molecular signals. Differentiation can be promoted by adding chondrogenic cytokines and growth factors to the construct. Some of the major players in the intricate network of chondrogenic cell signalling are well described in the literature, but other members are only known in very little detail or not at all. There is no question we still have much to learn, before we fully understand the temporal and spatial importance of the cytokines and growth factors involved in cartilage synthesis. However, studies have shown that even a simpler version with addition of only a few key molecules will strongly increase the quantity and quality of cartilage-like tissue produced.
In the following sections, we will present some of these key factors currently being used in cartilage engineering, and the delivery methods available.

4.1 Growth factors

4.1.1 Transforming growth factor-β superfamily

The transforming growth factor-β (TGF-β) superfamily includes the TGF-β and the bone morphogenetic (BMP) proteins. The most well described family members in relation to cartilage tissue engineering are TGF-β_1, TGF-β_3, BMP-2 and BMP-7 (also known as osteogenic protein-1 (OP-1)), but other members may prove to be equally important.
TGF- β_1, TGF-β_2, and TGF-β_3 have all been shown to be chondrogenic in vitro on MSCs (Kulyk et al., 1989; Blunk et al., 2002). They are believed to be involved in the early stages of the differentiation process (Chen et al., 2009). In vitro they are known to stimulate synthesis of ECM including collagens, fibronectin (Ignotz and Massague, 1986), and proteoglycans (Chen et al., 1987), and decrease the expression of collagen type 1 in MSCs (Kurth et al., 2007). Studies on human MSCs have shown that TGF-β_2 and TGF-β_3 are more chondrogenic than TGF-β_1 (Barry et al., 2001; Chen et al., 2009).

Unfortunately, the very promising results with TGF-β from in vitro studies have not been reproduced in animal models. In mice and rabbits intra-articular administration of TGF-β has been shown to have a number of side effects including inflammation and osteophyte formation (Bakker et al., 2001; van Beuningen et al., 2000). These side effects appear to be linked to the non-cartilaginous tissues of the joint (Blaney Davidson et al., 2007a; Scharstuhl et al., 2002), and can potentially be prevented if the growth factor only comes into contact with the cartilage.

The BMPs involved in chondrogenesis include BMP-2, -3, -4, -5, -6, -7, and -9. They support chondrocyte phenotype and stimulate synthesis of proteoglycans (Li et al., 2003; Sailor et al., 1996). BMP-2, and -7 have been shown to be the more chondrogenic of the BMPs in MSCs in vitro (Majumdar et al., 2001; Sekiya et al., 2005; Fan et al., 2010), and both have shown promising results in animal models (Badlani et al., 2009; Blaney Davidson et al., 2007b). The chondrogenic effect of BMP-7 is synergistically increased when used in combination with TGF-β (Xu et al., 2006; Kim and Im, 2009) or insulin-like growth factor (IGF)-1 (Loeser et al., 2003; Chubinskaya et al., 2007a). BMP-2 is also a strong inducer of osetogenesis and is used clinically for bone fusion (Tang et al., 2011; Hamilton et al., 2011), which needs to be taken into consideration if using BMP-2 for cartilage generation. Only BMP-2 and BMP-7 are currently approved for clinical use in human patients (Haleem and Chu, 2010).

In addition to the TGF-βs and BMPs, this large family of molecules also includes activin and growth differentiation factor 5 (GDF5) that have been shown to be chondrogenic inducers in MSCs (Jiang et al., 1993; Bai et al., 2004).

4.1.2 Insulin-like growth factor-1

IGF-1 is primarily synthesized in the liver regulated by growth hormone (GH). The responsiveness of cells to IGF-1 decreases with age and osteoarthritis (Boehm et al., 2007; Loeser et al., 2002). IGF-1 is capable of inducing chondrogenesis in MSCs on its own, but it is much more effective in combination with other growth factors e.g. TGF-β (Longobardi et al., 2006) and BMP-7 (Loeser et al., 2003; Chubinskaya et al., 2007b).

4.2 Delivery methods

Most growth factors and cytokines have short half-lives (Nimni, 1997), and their effect would therefore be limited to the time right after implantation of the construct. Subsequent supplementary treatments entail several risks depending on the choice of route of administration.

Systemic delivery presents a number of potential problems. Since the amount of active reagent actually reaching the site of interest will be a fraction of the initial dose, the administered dosage will have to be up regulated. This will increase both the cost of treatment and the risk of adverse reactions. In route to the target site the growth factors and cytokines will come into contact with a number of other tissues, where they may cause undesirable effects. Local injection into the construct provides a more controlled administration, but repeated joint injections are not desirable due to the risk of infection and added cost to the patient.

Because of these issues, a number of delivery systems have been developed and tested, where growth factors and cytokines are released into the local environment of the construct in a time and dose controlled manner. Some of these delivery systems are closely linked to the scaffold materials, and may not work well with all types of scaffolds. Other systems are

linked to the cells. A requirement for the successful delivery system is that the active molecules are protected against degradation until time of release. The systems range from very basic soaking of the scaffold in growth factor suspension (Kanematsu et al., 2004) to highly sophisticated release systems, where attempts are being made to closely mimic the stage specific differentiation process or even create dual tissues by releasing different molecules at controlled time points, dosages, and locations in the scaffold (Wang et al., 2009; Suciati et al., 2006). These highly complex systems are still in their infancy.

4.2.1 Direct attachment or incorporation

The simplest version of delivery system is a direct attachment of growth factor to the surface of the scaffold material by soaking the scaffold in growth factor suspension (Kanematsu et al., 2004), or incorporation of the growth factors and cytokines into biodegradable scaffolds, usually hydrogels (Yamamoto et al., 2001; Nelson et al., 2011). The factors are then released passively from the surface of the scaffold or as the scaffold degrades. Current applications of hydrogels primarily include drug delivery, since the scaffolds are too soft to play the structural role needed in cartilage constructs (Woodfield et al., 2002).

Molecule bound. Another way of incorporating growth factors and cytokines in scaffolds is to bind them to intermediary components in the construct. This method may prolong the effect of the growth factors by slowing down the release process. A study in rabbits using MSCs, elastic block copolymer scaffolds and TGF-β_3, showed that chondroitin sulphate-bound TGF-β_3 had a slower release profile than TGF-β directly incorporated into the scaffold (Park et al., 2010). Similar results have been achieved by binding TGF-β_3 (Park et al., 2008) or bFGF (Jeon et al., 2006) to heparin.

Loaded structures. The cytokines can also be delivered to the constructs in loaded structures. Microspheres (Kim et al., 2003; Elisseeff et al., 2001; Fan et al., 2007; Fan et al., 2004), liposomes (Hunziker et al., 2001), and micro sponges (Fan et al., 2010) loaded with growth factors have all been tested in studies in cartilage tissue engineering. Their advantage is a more controlled release rate, while the growth factors are kept relatively protected from their surroundings thus preserving their activity.

Gene therapy. A different approach to delivery of growth factors and cytokines important to chondrogenesis is to make the MSCs themselves produce the factors necessary to the cartilage construct. This can be achieved by using gene therapy techniques. The use of gene therapy in cartilage tissue engineering has been thoroughly reviewed by Steinert et al. (2008). Here, we will simply provide a short introduction to the concept. There are several different methods available ranging from a simple direct delivery of genetic material at the defect site to complex procedures implemented as part of the cartilage engineering process. No matter which method is used, the most important factors in gene therapy are related to how well the gene material is transferred to the target cell, and how efficient the now transgenic cells are at producing the desired molecules.

The gene therapy techniques fall into two categories – in vivo and ex vivo techniques. Common to them is the need for good vectors. These can be non-viral or viral. The non-viral vectors are safer but less efficient, while the viral vectors are more efficient but pose a potential safety risk especially if they are injected into the patient (Steinert et al., 2008).

In vivo delivery is the cheaper option, where vectors harbouring the gene material are introduced to the cells directly at the site of injury. This method is simple and fast, but it is difficult to control the efficacy of transfer as well as the safety of the procedure. Especially if

viral vectors are used there is a risk of the vectors inserting themselves into the genetic material of host cells in the area. The in vivo method is particularly useful in tissues where it is not possible to remove cells for ex vivo transfection. Its use in studies on cartilage repair has been very limited. The ex vivo method is more time consuming, expensive, and technically challenging. The genetic material is transferred to the cells, before the cells are used in the patient. Using this method makes it possible to test transfection rate, and the risks associated with use of viral vectors are eliminated. Ex vivo gene transfer has been tested in a number of studies involving MSCs and cartilage tissue engineering. MSCs transfected with BMP-7 yielded better cartilage repair than non-treated control cells (Mason et al., 2000). Similarly, gene induced expression of TGF-β1 and BMP-2 promoted chondrogenesis in MSCs, while induced expression of IGF-1 did not (Palmer et al., 2005). A subsequent study from the same group showed that the use of combinations of those three chondrogenic genes had a strong synergistic effect on chondrogenesis (Steinert et al., 2009).

5. Mechanical stimuli and bioreactors

During daily activities, the cartilage is exposed to direct compression, hydrostatic pressure, or shear. It has been suggested that mechanical loading may increase extracellular matrix (ECM) synthesis during cartilage engineering (Portner et al., 2009). The hypothesis that mechanical stimulation enhances cartilage formation is based on studies of developmental biology where restriction of joint loading after birth leads to poor post-natal cartilage adaptation (Williamson et al., 2003a; Williamson et al., 2001; Williamson et al., 2003b; Mikic et al., 2004; Mikic et al., 2000). Overall, mechanical stimulation leads to increased cell expansion as well as increased extracellular matrix proteins production compared to regular static cultures (Portner et al., 2009).

A bioreactor is defined as any device in which a biological/biochemical process is performed under controlled conditions. When compared to static cultures, bioreactors offer a number of advantages such as uniformed distribution and increased mass transfer, control of pH, temperature, gas supply (O_2 and CO_2), nutrients, waste product removal, and the opportunity to incorporate mechanical stimuli. Detailed reviews on the general concepts of bioreactors for tissue engineering and the application of bioreactors for the purpose of cartilage engineering are available (Godara et al., 2008; Chen et al., 2006; Chung and Burdick, 2008; Portner et al., 2009; Haasper et al., 2008; Huang et al., 2010b; Schulz and Bader, 2007; Concaro et al., 2009; Grad et al., 2011). Selected physical outcome parameters used in cartilage engineering in defined and described in Text Box 1.

Bioreactors for cellular therapeutic use have being grouped into two categories. Bioreactors for tissue engineering and bioreactors for cell infusion therapies. However, the simpler systems used for infusion therapies have also been utilized for tissue engineering purposes due to ease and cost. Bioreactors for isolation and expansion of cells for cell infusion therapies are largely similar to or adapted from pharmaceutical monoclonal antibody production systems or industrial yeast-based methods. Spinner flasks and rotating wall vessels are examples of bioreactors for cell expansion (Concaro et al., 2009). In spinner flasks a magnetic stir bar moves the medium. The media movement provides the cell-scaffold constructs with nutrients and oxygen and facilitates waste removal by overcoming the normal diffusion limit of 100-200 μm. In this system a balance has to be struck between homogenous mass transfer including uniform pH gradient and shear gradients that can cause cell damage. Stirring at 50 rpm is a common starting point (Concaro et al., 2009). If the

speed of the rotating outer wall of rotating wall vessels is calibrated to the mass of the cell carrier constructs, then a microgravity environment is created where the cells are exposed to low shear stress and high mass transfer. However, hitting this "soft spot" of equilibrium can be challenging and failure hereof may lead to shear stress and constructs colliding with the walls, which adversely affect cell function (Concaro et al., 2009). However, rotating wall vessels have been utilized to study microgravity's effect on cartilage tissue engineering both on Earth and in space (Vunjak-Novakovic et al., 2002; Marolt et al., 2006). Marked differences where noted between cartilage engineered on Earth and in space, on Mir, confirming that physical forces modulate musculoskeletal tissue such as cartilage (Vunjak-Novakovic et al., 2002). Bioreactors for cartilage engineering have largely been used to evaluate response to compression, but bioreactors applying electrical fields, ultrasound, centrifugal forces, shear forces, perfusion of 3D constructs, tension of cell layers, hydrostatic pressure, and hydrostatic pressure with perfusion have also been reported (Schulz and Bader, 2007). These bioreactors are mostly custom made, but an increasing number of commercial bioreactors are becoming available for various purposes (Yeatts and Fisher, 2011). Comparison between research groups is therefore exceedingly difficult. One major concern with many of the studies is the validation of the bioreactor prior to use and the continued calibration. Thorough evaluation and validation of the bioreactors prior to and continuously through their use by applying objective measurable parameters is critical in order to evaluate the results and conclusions. Many of the bioreactors can only stimulate the tissue and have limited, if any, possibilities of sampling and analyzing the tissue or culture medium without terminating the culture process.

Most cartilage bioreactor studies have reported work using uniaxial direct compression (Grad et al., 2011). A general starting point for direct compression studies of cells adhered to a scaffold is 10% or 15% compression at a frequency of 1Hz (Grad et al., 2011; Terraciano et al., 2007; Mouw et al., 2007; Huang et al., 2004). Recently, mechanical stimulation showed improved cartilage formation of porcine chondrocyte cartilage constructs compared to static culture, but no difference was noted between perfusion and perfusion-compression constructs (Tran et al., 2011). In the compression group, constructs where cultured with perfusion alone at a flow rate of 0.5ml/min for the first week followed by 1 Hz sinusoidal unconfined compression, 4 hours a day, 5 days a week, starting with a load of 0.5 N until 20 N by the third week (Tran et al., 2011). Perfusion was maintained in this compression group for the 4-week duration of the study and compared to a control group of perfusion only. Biochemistry revealed a higher glycosaminoglycan (GAG) content, but a lower collagen content in the bioreactor construct compared to native cartilage. The discrepancy between GAG and collagen content could be due to enzymatic collagen degradation or simply reflect immature cartilage since rabbit studies have shown that the cartilage collagen network does not mature until 3 months of age (Julkunen et al., 2009; Riesle et al., 1998). Rabbit joint cartilage at birth is homogenous with collagen fiber alignment parallel to the surface, but as the cartilage matures the collagen fibers organize into the three zones seen in adult cartilage (Julkunen et al., 2011). However, the specific cartilage organization and biochemical content differ from joint to joint and even between opposing joint surfaces of the same joint (Julkunen et al., 2011). Recently, bovine bone marrow (BM) derived MSCs exposed to long-term dynamic compression in chondrogenic culture were shown to exert improved mechanical properties compared to static culture as previously reported in studies of chondrocyte cultures (Huang et al., 2010a). Interestingly immediate mechanical stimulation of cartilage constructs appear detrimental and a more physiological approach of initial stem

cell chondrogenic differentiation using TGF-β without mechanical stimulation, as in utero, followed by mechanical stimulation, as post-natal, appear more effective (Terraciano et al., 2007; Mouw et al., 2007; Thorpe et al., 2008). These observations would concur with the observations of normal rabbit joint cartilage development as described above, where the cartilage at birth is a homogenous structure that remodels post-natally in response to loading.

Hydrostatic pressure in cartilage tissue engineering has been reviewed elsewhere (Elder and Athanasiou, 2009). Hydrostatic pressure does not exert any measurable strain on the cell and the mechanistic effect are believed to be mediated through deformation of transmembrane ion transport proteins leading to increased intracellular calcium concentrations (Schulz and Bader, 2007; Kornblatt and Kornblatt, 2002; Elder and Athanasiou, 2009). The effect of hydrostatic pressure is incompletely understood, but both dynamic and static pressure has been investigated in both normal chondrocytes, osteoarthritic chondrocytes and as a mean for chondrogenic cell differentiation (Elder and Athanasiou, 2009). Physiological hydrostatic pressure of 5-10 Mpa are generally anabolic when applied in a dynamic fashion to both 2D and 3D chondrocyte layers, but if static pressure is applied anabolic effect is only noted in 3D layers (Elder and Athanasiou, 2009). Such physiological loads can also promote stem cell differentiation, chondrocyte redifferentiation, and exert chondroprotective effects and osteoarthritic chondrocytes (Elder and Athanasiou, 2009). Super-physiological pressures (20-50 Mpa) are particular detrimental to cell metabolism if applied for more than 2 hours (Elder and Athanasiou, 2009). Schulz and Bader have reviewed the mechanism of hydrostatic pressure stimulation and why prolonged loading leads to cartilage damaged (Schulz and Bader, 2007). Interestingly, compression likely acts through hydrostatic pressure as well. The reason is that negatively charged proteoglycans provide frictional resistance by binding water and preventing the water from being squeezed out of the tissue. The net effect is increased hydrostatic pressure within the tissue. However, the water does shift out of the tissue if the pressure persists. In this case, the load is increasingly carried by the collagen fibers until the fibers are orientated parallel to the load direction. Load beyond this point leads to tearing of the collagen network.

One trend in cartilage bioreactor design is towards a more tribological approach, where the science and technology of interacting surfaces in relative motion is used to develop multi-axial bioreactors that more closely mimic the movement of a natural joint (Figure 4) (Grad et al., 2011). Multiaxial compression and shearing stimulation represent more closely in vivo conditions and are associated with broader chondrogenic gene expression profiles compared to uniaxial compressive loading (Grad et al., 2011). Another trend is the ability of bioreactors to simultaneously provide mechanical stimuli and evaluate the physical properties of the developing tissue (Portner et al., 2009). The ability to monitor the construct as it develops allows for a reduction in sample size since temporal changes can be assessed on the same sample. It may even be possible to perform online temporal molecular evaluation of the cells, the conditioned culture medium and extracellular matrix composition and responses in closed bioreactor system through the application of high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR), matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS), combinatory phase sensitive scanning acoustic microscopy (PSAM), and confocal laser scanning microscopy (Schulz and Bader, 2007). Such advanced bioreactors can be valuable tools in preconditioning cell-scaffolds prior to clinical research use as well as model systems

for in vitro evaluation of cell response to a variety of physical as well as chemical stimuli. These systems may also have value as model systems to reduce the number of animals used for in vivo studies by allowing replacement and refinement prior to in vivo studies. However, their utility for commercialization of tissue replacement strategies may be hampered by high production costs and violation of GMP due to the often semi-sterile productions systems.

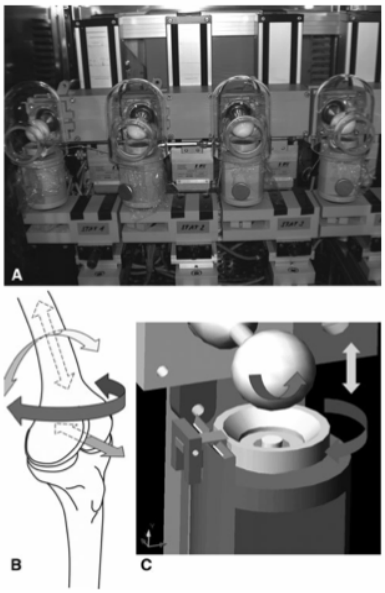

Fig. 4. Advanced bioreactor. (A) Advanced tribological bioreactor capable of multiaxial stimulation of tissue-engineered constructs. Each chamber can be individually controlled to simulate the movements of complex joints, e.g. the knee (b and C) Reprinted with permission from Grad et al. (2011)

Soft lithography produced microfluidic bioreactors have been advocated as the next generation of tissue engineering bioreactors to gain mechanistic insights due to affordability, flexibility and precision (Godara et al., 2008). Mesenchymal stem cells proliferation, motility and osteogenic differentiation in response to various culture regimes was studied using time-lapse imaging of 96 culture chambers within a single microfluidic chip (lab-on-a-chip) (Gomez-Sjoberg et al., 2007). However, one of the challenges is absorption of small hydrophobic molecules, typically hormones, by the plastic used for producing the microchips (Gomez-Sjoberg et al., 2010). Such methods may lend themselves well to studying the issue of MSC population heterogeneity, incomplete MSC chondrogenic programming, evaluation of novel chondrogenic inducing small molecules and signaling pathways involved in chondrogenic differentiation as identified elsewhere (Huang et al., 2010c). A potential limitation of such micro-bioreactors is that they mainly evaluate 2D structures and cartilage tissue tends to prefer a 3D environment.

As outlined, sophisticated bioreactors are currently available in a few labs for the generation and real-time analysis of cartilage constructs. However, the mechanical properties of the

produced cartilage remain significantly inferior to that of native cartilage although histological, biochemical and gene expression assays may indicate otherwise. A major drawback of most publications to date is the omission of a relevant positive biological control sample such as native cartilage. Most studies compare the test sample to a negative control sample consisting of the empty cell scaffold/carrier. Such study designs make it difficult to determine the biological relevance/utility of the engineered tissue. This being said, it is probably unknown when an engineered tissue is "good enough" for clinical implantation. This will likely depend on the constructs used as well as the patient age, lesion location and severity, and the accompanying rehabilitation program. Mature native tissue of the tissue of interest is likely the best biological yardstick presently, but the degree of tissue maturation preferable at the time of transplantation remains undetermined.

As alluded to already, another major challenge is the development of aseptic, cost effective, bioreactor systems that will fulfill current regulatory requirements. A significant segment of the bioreactor industry is dedicated to the development of disposable systems as reviewed elsewhere (D'Aquino, 2006; Eibl and Eibl, 2009; Singh, 1999). These systems include wave-mixed, orbital shaken and stirred bioreactors (Eibl et al., 2010). These systems may provide cost-effective solutions due to savings on operative utility, cleaning and validation costs as well as reduced water and cleaning agent consumption for cleaning. Some of the challenges are real-time analysis of the process due to range limitations of disposable sensors, pre-validation that would shift the regulatory burden from the end-user to the manufacturer, incompatibilities with certain chemicals and temperatures, and limited accessories such as valves and sampling systems (Eibl et al., 2010; D'Aquino, 2006). These systems have not yet been significantly evaluated for cartilage engineering.

De novo tissue engineering has also been investigated, where the so-called cell niche is relied upon to direct transplanted cells or tissue pieces towards the appropriate tissue has also been investigated (Grad et al., 2011; Shah et al., 2010; Stevens et al., 2005). This approach is often autologous in nature and associated with minimal handling and laboratory exposure of the cells. ACI and MACI are examples of this strategy. However, the resulting repair tissue remains inferior to native cartilage. Recently, self-assembling nanofibers were shown to promote cartilage repair of full thickness chondral defects in rabbits (Shah et al., 2010). Alternatively, tissue can be made in vivo at a site distant to the ultimate repair site for later relocation to the injury site. Such de novo tissue formation was elegantly demonstrated for bone regeneration in a rabbit model (Stevens et al., 2005). Bone formation was induced by alginate injection under the tibial periosteum and later the neo-bone was removed and transplanted into an induced cortical bone defect where it promoted bone healing. Cartilage tissue was also generated using this model by adding molecules to the gel that inhibited angiogenesis and promoted chondrogenesis.

The influence of oxygen tension during cell and tissue culture has also been investigated in relation to cartilage engineering and is an area, which appears to deserve further investigation. Low oxygen levels have been reported to be a more potent promoter of chondrogenesis than dynamic compression (Meyer et al., 2010). Previous studies have shown controversial findings in relation to the effect of oxygen tension on chondrogenic differentiation of mesenchymal stem cells with some reporting increased proliferation rates and chondrogenic potency and others reporting reduced proliferation and differentiation potency (Grayson et al., 2007; Merceron et al., 2010; Krinner et al., 2009; Holzwarth et al., 2010).

6. Pre-clinical animal models for safety and efficacy evaluation of engineered cartilage

Cartilage defects are "quality-of-life" lesions, as opposed to life-threatening conditions, for which therapies are available today that provide palliative relief for a large number of patients. Since long-term safety of new cell-based treatment modalities have yet to be determined it is possible that adverse effects could ultimately lead to worse quality of life than the initial cartilage problem caused. Thorough evaluation of efficacy and long-term safety is therefore prudent before introducing new cell-based therapies for such lesions. In vivo studies can generally be categorized into models where the animal is used as the bioreactor directly at the injury site or at a distant site for later relocation to the injury site, as discussed above, or as models were animals are used to evaluate in vitro engineered constructs.

Members of the Orthopaedic Trauma Association (OTA) recently discussed pre-clinical animal models for cartilage and bone repair (Lansdowne, 2010; Martineau, 2010). This topic has also been reviewed by a number of authors (Pearce et al., 2007; Reinholz et al., 2004; Chu et al., 2010; Koch and Betts, 2007). The consensus with regard to cartilage defects is that the research question determines the choice of model, since there is no ideal pre-clinical animal model. General issues to consider are the lesion model (chondral, osteochondral, degenerative), most appropriate model (cost, availability, joint structure, age), cartilage similarity (thickness, structure, cell density, biochemistry, biomechanics), and the nature of the lesion (area, depth, location) (Martineau, 2010). Small animal models are rodents and rabbits. Rodent models have limited translational value, but are cost effective models for mechanistic studies of chondrogenesis, generation of proof-of-concept data and bridging in vitro and large animal studies. Rabbits are easy to handle and cost effective, but have thin cartilage with excellent endogenous repair potential as well as highly flexible joints which support a relatively low body weight (low loading of the joints). Large animal models include minipigs, goats, and horses (Martineau, 2010). Mini-pigs do not comply with rehabilitation programs, but advantages comprise joint size sufficiently large to allow arthroscopy, partial or full-thickness chondral defects, growth plate closure, poor endogenous chondral repair potential and the possibility of making 6-8 mm diameter size lesions. Goats (as well as sheep) are similar to mini-pigs in that they do not comply with rehabilitation programs, but allow arthroscopic approaches and large defects can be made in cartilage that have limited intrinsic repair potential. Goats and sheep studies are more expensive due to increased housing and handling costs. Horses can comply with rehabilitation programs, allow for defect sizes similar to human defects, exert low intrinsic repair potential and arthroscopic treatment modalities and make follow-up assessment possible. The drawback of equine studies is high cost, high loading forces, and hard bone, and it is difficult to achieve protected weight bearing in the horse.

The OTA study group on bone defect models had the following universal considerations: consider the 3R's of animal use and reduce, refine and replace before choosing the most relevant animal model; perform a pilot study if inexperienced with the model; animal models do not account for co-morbidities (e.g. obesity, diabetes etc); ensure optimal animal care before and after surgery by consulting with veterinary specialists in surgery (www.acvs.org or www.ecvs.org), anesthesia (www.acva.org or www.ecva.eu.org), and laboratory animal medicine (www.aclam.org or www.eclam.org); ideally standardize your

model with a negative and a positive control although in comparative studies comparison with the current "golden standard" treatment is sufficient (Lansdowne, 2010).
The argument for increased use of domestic animal species as pre-clinical animal models is based on the fact that many human conditions have a spontaneous counter-part in animals that would allow for more physiologically relevant studies compared to induced lesions in inbreed subpopulations of small laboratory animals. Veterinary medicine today often rivals human medicine with regard to diagnostic and treatment modalities, some companion animals largely live in the same epigenetic environment as humans, and the veterinary market is of significant monetary value in itself (Koch et al., 2009; Koch and Betts, 2007). Most recently, induced pluripotent stem cells (iPS cells) have been generated from equine cells for the purpose of treating sporting horses as well as utilizing the horse as a pre-clinical animal model of comparative human disorders (Nagy et al., 2011). Stem cells and animal therapies have recently been reviewed elsewhere (Figueroa et al., 2011). The sentiment from 2004 by Fiester and colleagues that successful treatment of a spontaneous animal disorder may significantly advance stem cell research remains valid today and could be expanded to include the field of regenerative medicine (Fiester et al., 2004).

7. Conclusion and future perspectives

The field of cartilage tissue engineering is a very diverse research field investigating the use of bioreactors, scaffold compositions and designs in combination with a wide range of cells and cytokines. It is still an emerging research field and careful pre-clinical assessment of the potential treatment modalities is advised to avoid long-term adverse effects (Figure 5). However, these treatment modalities hold the potential of providing life-long solutions to the currently incurable problem of joint cartilage damage. Domestic animal models may provide valuable translation significance between proof-of-principle studies in small laboratory animals and expensive human pre-clinical trials.

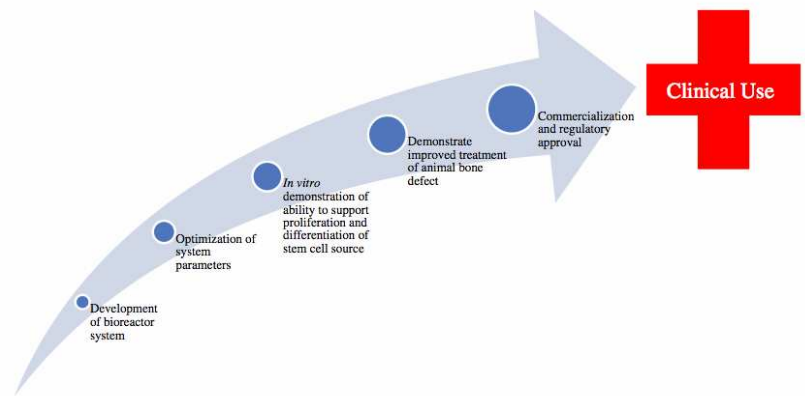

Fig. 5. Clinical roadmap for bone tissue engineering bioreactors. This roadmap is equally useful for cartilage engineering. In vivo proof-of-principle can be shown in laboratory animal models, but domestic animal models may be of more translation value prior to human clinical trials. Reprinted with permission from Yeatts and Fisher (2011)

Text Box 1. Selected physical outcome parameters used in cartilage engineering
Stress, strain and frequency are often referred to in tissue engineering so a brief description of these terms is warranted. Stress, often denoted as sigma (σ), is the amount of force acting over a given cross-sectional area. Stress is expressed as force per area units. The unit of stress is pascal (Pa) or newtons (N) per area in the metric system, and psi in the English system. Conversions of these different units are as follows: 1 psi = lb_F/in^2, 1 Pa = 1.45 x 10^{-4} psi, 1 Pa = 1 N/m^2, 1 kPa = 1 where Newton (N) is the SI unit of force. 1 N = kg m/s^2, e.g. the net force required to accelerate a mass of one kilogram at a rate of one meter per second squared. Engineering strain, often denoted as ε, is the nominal change in length of a material ($\varepsilon = \Delta L/L_0 = (L - L_0)/L_0$). The unit of strain is often mm/mm, cm/cm, etc, or no unit at all since it is the ratio of a given measuring system. Frequency, denoted with the SI unit hertz (Hz), is the number of cycles per second, typically of a sine wave. Shear stress, τ, as exerted on cells in perfusion systems with a laminar flow profile between the ingress and egress plates can be calculated as follows: $\tau = (6\mu Q)/(bh^2)$, where μ is the viscosity of the medium, Q is the volumetric flow rate, b is the width of flow channel, and h is the distance between the two plates (Shiragami and Unno, 1994; Nagel-Heyer et al., 2005). The shear stress unit is force per area, e.g. N/m^2 or similar representation of force per area using psi, Pa and in^2 or cm^2. Other physical parameters often used in cartilage engineering such as Darcy's law, Reynolds number (Re), mass flow rate, flow velocity has been reviewed elsewhere (Concaro et al., 2009).

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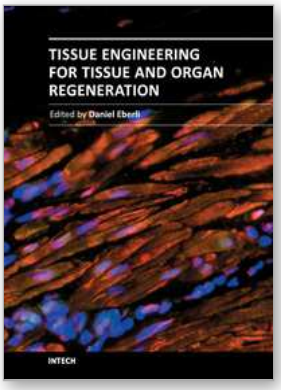

Tissue Engineering for Tissue and Organ Regeneration

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

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