Recent Advances in Cartilage Tissue Engineering: From the Choice of Cell Sources to the Use of Bioreactors*

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Grafting engineered cartilage tissues represents a promising approach for the repair of joint injuries. Recent animal experiments have demonstrated that tissues engineered by culturing chondrocytes on 3D scaffolds in bioreactors provide functional templates for orderly repair of large osteochondral lesions. To date, however, a reproducible generation of uniform cartilage tissues of predefined size starting from adult human cells has not been achieved. In this paper we review some of the recent advances and challenges ahead in the identification of appropriate (i) cell sources, (ii) bioactive factors, (iii) 3D scaffolds and (iv) bioreactors for human cartilage tissue engineering. We also present an example of how integrated efforts in these different areas can help addressing fundamental questions and advancing the field of cartilage tissue engineering towards clinical use. The presented experiment demonstrates that human nasal chondrocytes are responsive to dynamic loading and thus could be further investigated as a cell source for implantation in a joint environment.

Key Words: Tissue Engineering, Cartilage Repair, Cell Source, Bioactive Factor, Polymer Scaffold, Bioreactor, Mechanical Loading

1. Introduction

Articular cartilage is a highly specialized tissue that serves as a load-bearing material of diarthroidal joints, with excellent friction and wear characteristics. It is composed of one single type of highly specialised cells, the chondrocytes, embedded within a dense extracellular matrix (for a review, see Ref. (1)). The extracellular matrix is mainly composed of type II collagen and proteoglycans which give the tissue its form and stability. In particular, proteoglycans mainly confer resistance to compression, by attracting a large amount of water into the

intramolecular and intermolecular space, whereas collagen fibrils withstand the swelling forces of the water-proteoglycan phase and provide the matrix with high tensile strength. Thus articular cartilage, although it is only a few millimeters thick, ensures to the joint smooth gliding motion, load transmission, force distribution and minimises peak focal stress on the subchondral bone⁽²⁾.

Traumatic cartilage lesions affect mostly young subjects often involved in sustained sport activities⁽³⁾. These pathologies result in severe pain and disability for millions of people world-wide and represent a major challenge for the orthopaedic community. Focal cartilage defects are classified as either chondral or osteochondral. *Chondral lesions* lie entirely within the cartilage and do not penetrate beyond the calcified zone into the subchondral bone. The vasculature being unreachable, these lesions do not heal

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spontaneously and are analogous to those observed during the early phases of osteoarthritis^{(4),(5)}. *Osteochondral defects* penetrate the articular surface in depth through the vascularized subchondral bone. Some spontaneous attempts of repair can be observed as mesenchymal chondroprogenitor stem cells invade the lesion, differentiate into chondrocytes and form cartilage⁽⁶⁾. However, repair of full-thickness defects ends up to be only transient: the repair tissue is fibrous in nature, does not have the functional properties of native hyaline cartilage, and thus results more vulnerable to the action of free radicals, metalloproteinases and catabolic cytokines including IL-1 and TNF- α ^{(7),(8)}. Matrix breakdown often leads to evident clefts and fissures on the articular surface and subsequently to the onset of degenerative joint diseases such as osteoarthritis⁽⁹⁾.

It is thus clear that articular cartilage has a limited capacity for self-regeneration after injury. Intrinsic biological features of the tissue itself are the limiting factors: 1) mature chondrocytes have a relatively low metabolic activity and proliferation capacity and the abundant extracellular matrix may provide a barrier for their migration to the defect; 2) articular cartilage being neither vascularized nor penetrated by lymphatic vessels, there is no direct access to progenitor cells; furthermore, the supply of growth and differentiation promoting factors is ensured only by diffusion from the synovial fluid; 3) proteoglycans of the extracellular matrix have anti-adherent properties and may prevent cell adhesion, further undermining any tentative repair process⁽¹⁰⁾; herein may also lie the difficulty of achieving a good integration between repair tissue and native cartilage; 4) finally, articular cartilage is not innervated and thus insensitive to early injuries that can readily progress to degeneration much before evident clinical symptoms emerge. Total joint replacement using artificial prostheses generally eliminates pain and partially restores functionality, but is limited by the durability of the device. Thus, particularly for young individuals, it is mandatory to identify alternative procedures to permanently repair cartilage injuries or at least to delay the implantation of an artificial joint replacement.

A large repertoire of most diversified therapeutic approaches has been developed to repair cartilage injuries. None of the traditional surgical methods (e.g., debridement and subchondral drilling) is capable of reproducibly ensuring long-lasting regeneration, and the use of allografts/autografts is limited by the availability of donor tissue and/or morbidity at the donor site. A major breakthrough in the field has been introduced by the procedure called autologous chon-

drocyte implantation (ACI), consisting in isolating and expanding chondrocytes from a small cartilage biopsy and injecting them under an autologous periosteal flap, used to confine the volume of the lesion. From a surgical standpoint, the main limitation of ACI is related to the mechanism of graft fixation. It was shown that the periosteal flap delaminates regardless of the postoperative rehabilitation protocol, and therefore the control over the fate of the injected cells is poor. This problem becomes even more relevant when large osteochondral defects are to be treated.

An innovative approach to cartilage repair is to generate *in vitro* functional cartilaginous constructs, where cells are embedded in a physiological extracellular matrix. As compared to cells injected under a periosteal flap or immobilised in a hydrated gel, implantation of structurally and mechanically functional cartilage tissues would allow easier graft fixation, early recovering of joint functionality and reduced mechanical discontinuity on the articular surface, which in turn might account for improved durability of the repair. Pre-formed cartilage could ultimately be combined with osteoconductive materials in order to generate osteochondral grafts.

2. Engineered Cartilage Grafts for the Repair of Large Joint Injuries

In the past few years, a cell-polymer-bioreactor system has been developed for the engineering of cartilage tissues of predefined size and shape⁽¹¹⁾ (Fig. 1). Primary bovine chondrocytes are first seeded into biodegradable non-woven meshes made of polyglycolic acid (PGA), which provide a 3D scaffold for the deposition and accumulation of extracellular matrix. The resulting cell-polymer constructs are then cultured in bioreactors applying dynamic laminar fluid flow, in order to increase rates of mass transfer. Engineered cartilage generated in this system has been reported to approach the biochemical and

Fig. 1 A cell-polymer-bioreactor system for the generation of functional engineered cartilage tissues, as described by Freed et al.⁽⁵⁰⁾

Fig. 2 Repair of large osteochondral defects in adult rabbits by implantation of an osteochondral composite, consisting of a layer of engineered cartilage combined with an osteoconductive support (Collagraft) (See Schaefer et al.⁽¹³⁾ for details on the study). After 6 weeks (A), the original shape of the engineered cartilage (ec) was still preserved, on top of the Collagraft material (cg). After 6 months (B), the engineered cartilage remodelled into a cartilaginous surface, matching the thickness of native rabbit cartilage, and new trabecular bone. A clear tidemark was separating the trabecular bone (C, von Kossa stain) and the cartilage surface (D, type II collagen immunostain). Scale bar = 2 mm (A, B) or 0.2 mm (C, D).

electromechanical properties of native bovine cartilage⁽¹²⁾, and thus represents an ideal candidate graft for implantation in a cartilage defect.

A recent study reported the implantation of engineered cartilage in large osteochondral defects in rabbit joints⁽¹³⁾. A layer of functional cartilage, generated by bioreactor culture of chondrocytes seeded onto PGA meshes, was combined with an osteoconductive support made of a ceramic/collagen sponge (Collagraft). The composites were press-fit in the femoropatellar groove of adult rabbits, in the largest ever created defects (7×5×5 mm) (Fig. 2). The engineered cartilage, which was 5 fold thicker than the native rabbit cartilage, withstood physiological loading immediately after implantation and remodelled within 6 months into osteochondral tissue consisting of a cartilaginous surface and new trabecular bone that was well anchored to the host bone. The cartilage surface contained evenly distributed GAG and type II collagen and displayed characteristic architectural features, uniform thickness as well as Young's moduli almost normal. Control defects left empty or treated with cell-free scaffolds healed with irregularly shaped fibrocartilaginous tissue. The study left many open questions, including the mechanism by which the cartilage was

remodelled, the origin of the cells forming the repaired cartilage surface (from the implant or from the host), and the long-term stability of the engineered cartilage. However, it demonstrated for the first time that functional engineered cartilage grafts, combined with an osteoconductive support, might provide a template permitting the orderly repair of very large osteochondral defects in adult rabbits. This approach therefore deserves further extension to a human cell source.

Despite all the recent progresses made in *ex vivo* synthesis of cartilage, a reproducible generation of uniform cartilaginous tissues of pre-defined size starting from adult human cells has not yet been achieved. We believe that this goal could be reached only by coordinated efforts in different areas, in order to identify: (i) an appropriate source of *chondrogenic cells*; (ii) the *bioactive factors* required by these cells during the different culture phases; (iii) the specific features of *3D scaffolds* in which cells can be grown; and (iv) the regime of physical stimulation that a *bioreactor* should apply to enhance cartilage development and maturation in a highly controlled environment. The following sections will introduce and discuss some of the main issues relative to these 4 different areas.

2.1 Cell source

For all cartilage repair methods based on autologous cells, a critical issue is related to the procurement of chondrogenic cells. In fact, a cartilage biopsy in the joint, necessary for autologous chondrocyte preparation, represents an additional injury to the cartilage surface, and has been reported to be detrimental to the surrounding healthy articular cartilage⁽¹⁴⁾. In addition, this approach is limited by the availability of cells, particularly in elderly individuals, and by their capacity to proliferate and to differentiate after expansion in monolayers. The first approach to overcome these drawbacks is to use different cell sources, including chondrocytes from non-articular cartilage types or mesenchymal progenitor cells.

Biopsies from the rib or the nasal septum can be removed under local anaesthetic and by a less invasive procedure than removing tissue from specific areas of the joint. Furthermore, since the donor site is not subjected to compressive forces, there is a minimal risk of morbidity at the biopsy site. It was recently shown that as compared to articular chondrocytes, human chondrocytes from the nasal septum proliferate approximately 4 times faster and have a higher capacity to generate a cartilaginous tissue after monolayer expansion⁽¹⁵⁾. However, extensive data from *in vivo* experimental studies will be needed to

demonstrate the efficacy of nasal chondrocytes at articular sites.

An alternative to the use of differentiated chondrocytes is the use of cells with chondrogenic differentiation capacity. Mesenchymal progenitor cells with a chondrogenic potential have been isolated from a variety of tissues, including periosteum⁽¹⁶⁾, bone marrow⁽¹⁷⁾ and synovial membrane⁽¹⁸⁾. Potential advantages of using mesenchymal progenitor cells as compared to differentiated chondrocytes include: (i) higher proliferative capacity, even from older individuals; (ii) higher responsiveness to growth factors and signalling molecules; and (iii) ability to differentiate into chondrocytes or osteoblasts according to the local environment. This latter possibility would allow the use of the same cell graft to repair both the articular cartilage and the subchondral bone.

There have been a few experimental studies on the repair of focal articular lesions using mesenchymal progenitor cells from bone marrow^{(19)–(21)} and from periosteal/perichondrial tissues^{(22),(23)}. One of the earliest study⁽¹⁹⁾ highlighted the possibility that the cartilage regenerated in osteochondral defects by marrow-derived mesenchymal progenitor cells could get thinner and fibrillated after prolonged time, consistent with the known characteristic of these cells to terminally differentiate *in vitro* into hypertrophic chondrocytes. In general, the data reported are qualitative and no study has shown yet either consistent results or the generation of a functional tissue comparable to native hyaline cartilage. In addition, no report has been published so far regarding the clinical use of mesenchymal progenitor cells from any tissue source for articular cartilage repair. Thus, to date these cells remain an unrealised promise for effective regeneration of articular cartilage lesions⁽²⁴⁾.

2.2 Bioactive factors

It is long known that human articular chondrocytes can undergo only a limited number of cell divisions *in vitro* and that their proliferative potential decreases with age⁽²⁵⁾. In addition, the redifferentiation of expanded human chondrocytes was reported to be negligible⁽²⁶⁾, to decrease with serial passaging⁽²⁷⁾, to fully develop only in long-term cultures^{(28),(29)}, and to be highly dependent on the lot of serum used⁽³⁰⁾. Limitations related to cell availability and capacity to proliferate and differentiate can potentially be overcome by using specific regulatory molecules during the different culture phases.

Specific growth factors (e.g., FGF-2 and TGF β 1) during articular chondrocyte expansion were recently shown not only to increase the cell proliferation rate, but also to maintain the ability of the cells to redifferentiate upon transfer into a 3D environ-

ment^{(31),(32)} and to respond to differentiating agents⁽³³⁾. At present, however, there is no comparative animal or clinical study reported in the literature concerning the use of articular chondrocytes expanded under conditions favouring cell proliferation and maintenance of chondrogenic ability.

The re-differentiation of human articular chondrocytes after monolayer expansion can also be supported by the use of different regulatory molecules during the phase of 3D culture. As an example, it was recently reported that arachidonic acid-derived lipids present in the synovial fluid, namely prostaglandins, can specifically modulate and in some cases (e.g., for prostaglandin F_{2a}) enhance chondrogenesis⁽³⁴⁾.

2.3 Three-dimensional scaffolds

Scaffolds used in tissue engineering of articular cartilage should display biological and physical properties matching both the needs of differentiating chondrocytes *in vitro* and of regenerating cartilage *in vivo*⁽³⁵⁾. Thus, the porosity of the scaffold should allow the exchange of nutrients and waste products, while providing a sufficient physical support for cells and extracellular matrix. The degradation rate of the scaffold should be tuned on the rate of production of extracellular matrix by chondrocytes, considering that the physical environment to which the scaffold is exposed *in vivo* may have an effect on its degradation. In addition, the mechanical characteristics of the scaffold should be such that at the time of implantation the cell-scaffold construct will display biomechanical properties compatible with those of the joint.

Different research groups have used a wide variety of scaffolds in the attempt to generate cartilaginous tissues *in vitro*. The form and composition of these scaffolds range from non-woven meshes and foams of alpha-hydroxypolyesters^{(36)–(40)}, polyglactin⁽⁴¹⁾ or hyaluronan alkyl esters^{(42)–(44)} to photocrosslinked hydrogels^{(45),(46)} and sponges based on different types of collagen and glycosaminoglycans^{(47),(48)}. Composites consisting of a 3D scaffold filled with cells embedded in a fibrin or alginate gel have also been explored, in order to combine the advantages of a predefined shape support and of media favouring maintenance of a rounded cell morphology⁽⁴⁹⁾.

Overall, the developed scaffolds have been tested with chondrogenic cells (mostly with non-expanded animal chondrocytes) and reported to support the formation of cartilage-like structures *in vitro*. However, fundamental questions related to understanding (i) the interactions between cells and specific substrates, (ii) the influence of the pore size distribution on cell behaviour, and (iii) the effect of scaffold geometry (i.e., in the form of a foam, mesh or gel) on

the induction/maintenance of the chondrocytic phenotype are yet to be answered. Efforts in this direction will be needed to identify objective criteria for the selection of scaffolds for the regeneration of cartilage lesions.

2.4 Tissue culture bioreactors

Engineering functional cartilage tissue may be enhanced by the use of bioreactors that provide a controlled *in vitro* environment over specific biochemical and physical signals known to regulate chondrogenesis. In particular, bioreactors could improve the formation of cartilage tissues by providing: (i) mixing patterns that result in efficient and spatially uniform seeding of three-dimensional scaffolds; (ii) enhanced mass transport of chemical species to the cells cultured into the scaffold; and (iii) physical (e.g., hydrodynamic, mechanical) stimulation of the cell-scaffold constructs during their development⁽⁵⁰⁾.

Uniform and efficient seeding of cells on porous scaffolds is an important prerequisite for the development of a homogeneous tissue from the lowest possible cell number. As compared to static loading of cell suspensions into scaffolds, seeding into orbitally mixed Petri dishes yielded thicker constructs with more spatially uniform distribution of cells and extracellular matrix⁽⁵¹⁾. The concept of maintaining a uniform suspension of isolated cells and providing a relative velocity between cells and the scaffold during seeding was extended by seeding in rotating vessels⁽⁵²⁾ or mixed flasks⁽⁵³⁾. Dynamic seeding using mixed flasks was shown to achieve seeding efficiencies approaching 100%, but led to cell densities higher at the scaffold periphery. In addition, the method is mostly suited to fiber meshes as opposed to porous foams. The design of bioreactors providing the possibility of scaffold perfusion with a cell suspension in alternate directions has the potential to yield a more homogeneous seeding on a variety of scaffolds⁽⁵⁴⁾.

Once the cells are associated with the scaffold, cell-polymer constructs can be cultured in bioreactors applying specific regimes of fluid flow. It was previously shown that dynamic (rather than steady) and laminar (rather than turbulent) flow patterns were associated with rapid *in vitro* assembly of functional cartilaginous matrix containing glycosaminoglycans and type II collagen by articular chondrocytes cultured on biodegradable scaffolds⁽¹²⁾. Such findings could be explained by the fact that dynamic laminar flow generated by rotating wall vessels provided efficient mass transfer, which was essential to promote or maintain cell differentiation, as well as synthesis and retention of extracellular matrix macromolecules.

Tissue engineering bioreactors can also expose

developing cartilage constructs to specific physical stimuli that may be beneficial for tissue growth and maturation⁽⁵⁵⁾. Enhanced chondrogenesis and cartilage formation were reported upon stimulation of (i) cartilaginous nodules by intermittent motion of medium in roller bottles⁽⁵⁶⁾, (ii) gel-immobilised chondrocytes by dynamic compression^{(57),(58)}, (iii) cell-polymer constructs by intermittent hydrostatic pressure⁽⁵⁹⁾, and (iv) chondrocyte monolayers⁽⁶⁰⁾ and cell-polymer constructs⁽¹²⁾ by hydrodynamic shear.

Despite the great efforts currently dedicated to the development and use of bioreactors for the engineering of functional cartilage tissue, it remains unclear which specific physical stimulation regimen is required to induce a specific effect on cultured chondrocytes. The scenario is further complicated by the fact that different scaffolds may transduce similar physical signals in different ways to cells, making it difficult to compare results obtained by different groups.

It is then obvious that advances in the generation of functional cartilage grafts prompts for tight interactions between scientists with different backgrounds, including medical doctors, cellular and molecular biologists, engineers, and physicists. The experiment presented in the next section aims at illustrating an example of how factors related to cells, bioactive molecules, polymer scaffolds, and bioreactors can be integrated to address fundamental questions in cartilage tissue engineering and possibly advance the field towards a clinical application.

3. Example of an Integrated Tissue Engineering Study: Are Human Nasal Chondrocytes Responsive to Dynamic Loading ?

As previously described (see Section 2.1), human chondrocytes from the nasal septum have a superior capacity to form cartilaginous tissues *in vitro* than articular chondrocytes⁽¹⁵⁾. In order to safely use implants generated by *nasal* chondrocytes for *articular* cartilage repair, it is however necessary to address the question of how nasal chondrocytes would behave in a joint environment, where they are exposed to a different physical environment as compared to that of the nasal septum.

As a preliminary investigation, in the experiment presented here we addressed the question of whether human nasal chondrocytes, associated with biodegradable scaffolds, are responsive to loading. In particular, we tested the hypothesis that, similarly to articular chondrocytes, also nasal chondrocytes are differentially responsive to static and cyclic loading, as assessed by changes in the mRNA expression levels of specific chondrogenic markers.

Fig. 3 Bioreactor for the application of compressive deformation to engineered constructs, consisting of: (I) compression chambers, (II) micrometer screws, (III) magnetic stirrers, (IV) positioning table, (V) load cell, (VI) water-cooled stepper motor. Two devices allow simultaneous application of static and dynamic compression.

3.1 Materials and methods

3.1.1 Chondrocyte isolation and expansion

Human adult nasal cartilage was obtained after informed consent from one patient undergoing nasal septum correction (male, 44 years old). Cartilage was minced finely and chondrocytes were isolated upon 22-hour incubation at 37°C in 0.15% type II collagenase. Cells were plated in culture dishes at 10^4 cells/cm² and expanded in Dulbecco's modified Eagle's medium containing 10% bovine serum and supplemented with specific growth factors (1 ng/ml TGF- β 1, 5 ng/ml FGF-2, 10 ng/ml PDGFbb), previously shown to enhance the proliferation rate and the re-differentiation capacity of human chondrocytes^{(15),(32)}. After 2 passages, cells were seeded into discs (5 mm $\times$ 2 mm thick) of non-woven meshes made of a hyaluronic acid-derived polymer (HYAFF-11®, Fidia Advanced Biopolymers, Abano Terme, IT) using spinner flasks stirred at 30 rpm and maintained in culture for 5 days prior to transfer in the bioreactor described below.

3.1.2 Mechanical loading Tissue specimens were cultured in circular chambers, in peripheral wells surrounding a central well containing a magnetic bar⁽⁵⁵⁾ (Fig. 3). The chambers were positioned on magnetic stirrers operating at moderate speed (30 rpm), so that nutrients in the medium were maintained at uniform concentrations. For the compressive deformation of the constructs, six plungers were connected to the cover lid of the chambers, in a position corresponding to the peripheral wells. The cover lid

of each compression chamber was connected to a positioning table coupled to a computer-controlled stepper motor. The contact between the plungers and the constructs was established using 6 micrometer screws coupled to the plungers and was verified by increases in the force measured by load cells. Two identical devices with two chambers each were built to allow simultaneously static and dynamic compression of constructs. Both devices were fit in an incubator at 37°C and 5% CO₂.

Cell-polymer constructs were maintained uncompressed (free swelling), or were exposed to static compression (25% strain) or to sinusoidal compression (25% strain offset, 20% strain amplitude) for 2, 14 or 24 hours. Triplicate specimens were assessed for each of the 9 experimental groups.

3.1.3 Analytical methods Using real-time quantitative RT-PCR, samples were assessed for the mRNA expression of collagen type II, aggrecan and chondromodulin (markers of chondrocytes differentiation), collagen type I and versican (markers of chondrocyte dedifferentiation), TGF- β 1 (marker of chondrocyte anabolic activity), and MMP-8 (marker of chondrocyte catabolic activity).

The expression levels of these genes were normalized to those of the housekeeping gene GAPDH. Values are presented as mean $\pm$ standard deviation. Statistical differences were assessed by one way analysis of variance and considered significant with values of $p < 0.05$.

3.2 Results

As compared to the free swelling controls, dynamic compression for 2 hours induced a significant increase in the mRNA expression of collagen type II, collagen type I, aggrecan, versican, chondromodulin and MMP-8 by respectively 9.1, 8.3, 6.4, 29.1, 4.4, 5.4 fold (Fig. 4A). Upregulation of collagen type II, aggrecan, versican, chondromodulin, TGF- β 1 and MMP-8 was also significantly higher in dynamically than in statically compressed constructs by respectively 7.1, 7.6, 6.8, 4.8, 2.2, and 3.2 fold. After 14 and 24 hours culture, the mRNA expression levels of all the genes were not significantly different in the 3 experimental groups and averaged the values of the free swelling condition after 2 hours culture (Fig. 4B, results at 24 hours not shown).

3.3 Discussion

The described experiment demonstrates that human adult nasal chondrocytes, expanded in the presence of growth factors and cultured in non-woven meshes based on hyaluronic acid, are differentially responsive to static and cyclic loading. In particular, upregulation of certain genes was specifically induced by the dynamic component of the compression. Inter-

Fig. 4 mRNA expression levels of collagen type II (CII), collagen type I (CI), aggrecan (Agg), versican (Ver), chondromodulin (ChM), TGF-beta1 and MMP-8 normalized to the housekeeping gene GAPDH in chondrocyte-polymer constructs cultured under free swelling, static or dynamic compression for 2 hours (A) and 14 hours (B). (*= statistically significantly different from the free swelling group; +=statistically significantly different from the static compression group).

estingly, such genes included markers of chondrogenic differentiation, as well as of chondrocyte anabolic and catabolic activity. Thus, longer-term experiments will be necessary in order to assess the precise phenotype reached by chondrocytes in response to loading and the quality of the resulting tissues. Since all the effects measured at 2 hours were not observed after a longer time of stimulation, we can speculate that chondrocytes desensitized to loading. Thus, with the goal of a sustained increased expression of extracellular matrix genes, which could lead to the engineering of more functional cartilage implants, we are currently investigating the effect of intermittent regimes of stimulation applied for longer periods of time.

4. Conclusion

In this paper we reviewed some of the factors relevant for tissue engineering of functional human cartilage grafts, relevant for the treatment of joint injuries. We also presented an example of how specific cell sources, bioactive factors, 3D scaffolds and bioreactors can be studied in an integrated fashion towards the generation of implantable tissues.

Once human engineered cartilage tissues are generated, a major challenge will still be represented by the integrative capacity of the implants. It has been described that cartilaginous grafts often do not

integrate with the host cartilage to form a continuous, mechanically stable attachment. Such lack of repair could lead to abnormal stress distribution during physiological activity and degeneration in the long term⁽⁶¹⁾. *In vitro* model systems have been recently developed to study factors affecting cartilage-cartilage integration without systemic effects and variability that are inherent to *in vivo* studies⁽⁶²⁾. Studies in this direction are likely to lead to innovative strategies for enhanced integrative cartilage repair.

Finally, in order to validate the efficacy of repair procedures based on engineered cartilage in the clinical setting and compare results with alternative methods, it will be necessary to perform rigorous controlled and randomised trials with stringent evaluation of the outcomes. Considering that the 'success' of any procedure aiming at the repair of cartilage injuries can only be quantified as the durability of a pain-free and functional joint, observation time in clinical trials should be extended for several years before any conclusion can be drawn. Since this fact represents a major obstacle in comparing different treatments within a reasonable time frame, strong emphasis should be put in the identification of early predictors of long-term outcomes, in order to allow establishment of shorter but equally relevant clinical trials.

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