Tissue engineering: advances in *in vitro* cartilage generation

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Damaged or diseased articular cartilage frequently leads to progressive debilitation resulting in a marked decrease in the quality of life. Tissue engineering, a budding field in modern biomedical sciences, promises creation of viable substitutes for failing organs or tissues. It represents the amalgamation of rapid developments in cellular and molecular biology on the one hand and material, chemical and mechanical engineering on the other. Current tissue engineering approaches are mainly focused on the restoration of pathologically altered tissue structure based on the transplantation of cells in combination with supportive matrices and biomolecules. The ability to manipulate and reconstitute tissue structure and function *in vitro* has tremendous clinical implications and is likely to have a key role in cell and gene therapies in coming years.

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Osteoarthritis (OA), the most prevalent disorder of the musculoskeletal system, is a consequence of mechanical and biological events that destabilize tissue homeostasis in articular joints. The disease process leads to joint pain, tenderness, limitation of movement, occasional effusion and variable degrees of inflammation. Prevalence studies indicate that the majority of people over the age of 65 have some OA. However, rheumatoid arthritis (RA) is a common form of inflammatory arthritis occurring in ~1% of the population and has the highest rate of onset in patients aged between 30 and 50 years.

The main pathologic features of OA are thought to develop as a result of dysregulation of tissue turnover in the weight-bearing articular cartilage and subchondral bone [1]. They are driven by local production of cytokines and proteases by the cells in the cartilage, synovium and bone. OA of knee, for example, is therefore biochemically mediated but it is probably mechanically driven-its localization depending on loading. It can be either caused by an age-related loss of ability of the tissue to respond to normal forces (primary OA) or the inability of the tissue to respond to excess loading (secondary OA).

RA is characterized by chondrocytes producing inflammatory signals such as interleukin 1 (IL-1), expression of matrix metalloproteinases (MMPs), decrease in production of MMP inhibitors and switch to a production of 'immature' matrix components typical of de-differentiated cells [2]. All these changes result in thinning of the collagen network, a decrease in the size of proteoglycan aggregates, loss of proteoglycans into the synovial fluid and reduction of biomechanical resistance. As a result, there is an influx of water, carrying cytokines or enzymes into the cartilage causing it to swell. In addition to this endogenous break down, some arthritic conditions are accompanied by a localized infiltration of synovial pannus tissue into the cartilage. Interactions between infiltrating cells and cartilage matrix or chondrocytes can be enhanced by stimulatory factors secreted at the pannus cartilage junction, at which activated chondrocytes might have a key function. Although there are differences in the histopathological features of cartilage destruction between OA and RA, some pathomechanisms that initiate the autodegradation of cartilage are thought to be shared in chronic joint destruction. Important degradation triggers include the cytokines tumour necrosis factor-α (TNF-α) and IL-1β [3].

Initial attempts for cartilage repair by autologous chondrocyte transplantation were presented by Brittberg *et al.* [4]. However, these procedures were performed exclusively in patients with cartilage injuries. The outcome of conventional surgical procedures for treatment of OA including joint resurfacing (abrasion, drilling, debridements, microfracture techniques or arthroscopic shaving) or biological autografts is unsatisfactory following long-term evaluation. This failure is caused by insufficient repair resulting in the formation of mechanically inadequate resident fibrocartilage. These disappointing results and the limited therapeutic opportunities have lead investigators to focus on more appropriate bioregenerative approaches, which could be specifically tailored for a patient's need.

Tissue engineering and bioregeneration approach

During the past decade exciting new strategies have emerged that have the potential to revolutionize the treatment of patients suffering from failure of vital tissue functions. The basic knowledge gained in the fields of cell and molecular biology, combined with the impact of biomaterial research, has provided a practical approach of bioregeneration. Tissue engineering procedures focus on the delivery or *in situ* mobilization of capable cells to restore pathologically altered architecture and function of tissues. This approach comprises the interactive triad of responsive cells, a supportive matrix and bioactive molecules promoting differentiation and regeneration [5] (Table 1).

Tissue engineering approaches are mainly focused on the restoration of pathologically altered tissues and organs based on the transplantation of cells in combination with supportive matrices and biomolecules. Development of vital transplants is simultaneously backed with new cell culture systems: complex 3D cell cultures in gels (e.g. collagen, agarose, alginate and fibrin) [6] or degradable polymer scaffolds within specific bioreactor modules. An elaborate cellular

Table 1. Developing procedures used in cartilage tissue engineering

	1st Generation (Present day)	2nd Generation (Just emerging)	3rd Generation (Future)
Principal approach	Autologous cell transplants	Preformed tissue flaps, 3D constructs and osteochondral transplants	*In vivo* regeneration and guided tissue repair
Important component	Periosteal flap	Delivery substances and scaffold	Growth factors and biomaterials

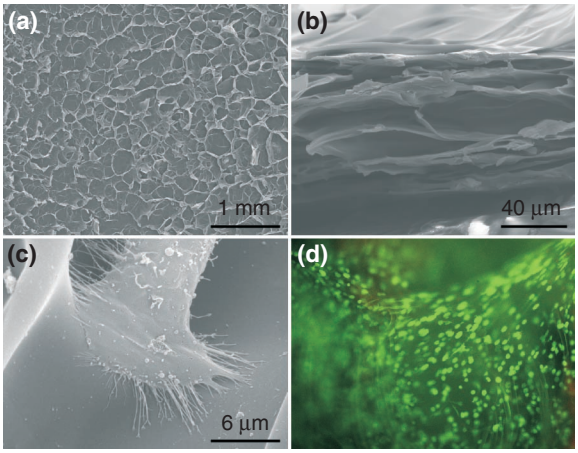

Fig. 1. Scanning electron micrographs of freeze-dried chitosan-gelatin scaffolds showing (a) Surface showing highly porous surface (scale bar = 1mm). (b) Cross sectional view of scaffold showing extensive interconnections (scale bar = 40 μm). (c) Micrographs showing a human nasal septal chondrocyte establishing contact with scaffold matrix (scale bar = 6 μm). (Adapted from [11], Reproduced, with permission, from Cognizant Communication Corporation, NY, USA). (d) Cell viability evaluation in engineered cartilage construct. Human chondrocytes growing in fibre fleece scaffolds. Viable cells are labelled green by fluorescein diacetate.

microenvironment is created that mimics the *in vivo* situation more closely than conventional cell cultures (Fig. 1). Mesenchymal cells, such as chondrocytes, in particular undergo a process of phenotypic and functional dedifferentiation when cultured in monolayer systems that lack the crucial influence of physiological cell–cell and cell–extracellular matrix (ECM) interactions. A growing body of evidence indicates that these interactions, which directly influence cell signaling via cell adhesion molecules such as integrins or cadherins, are of vital importance for nearly all cell functions [7]. 3D cell cultures provide the advantage of anchorage independent cell growth allowing cell motility, the synthesis of a specific pericellular or intercellular matrix and the physiological release and storage of bioactive molecules such as cytokines and morphogenic factors. During a 3D culture period, the quality of the tissue formed is influenced by the type of nutrient supply but is strictly dependent on factors that signal the differentiation towards a specific phenotype. These morphogens are even more essential for tissue engineering from precursor cells or mesenchymal stem cells. An increasing number of TGF-β superfamily members (bone morphogenic proteins, BMPs) are implicated in this process. These proteins, present in demineralized bone, induce differentiation of mesenchymal precursors to form cartilage [8]. More recent work demonstrates that individual factors exhibit individual as well as overlapping effects. This could depend on the complexity of homo- and heterodimerization of individual BMPs [9], the promiscuity of binding to different types of receptors, as well as activation of different intracellular signalling pathways [10]. These specific effects, which depend on the source of cells as well as the type, the concentration and the time of morphogen action is one of the most fascinating challenges for the engineering of human tissues.

Essential components of cartilage tissue engineering

Autologous chondrocytes

Tissue engineering protocols usually require handling of isolated autologous cells. Tissue samples from patients have to be isolated by enzymes such as collagenase and hyaluronidase to remove extracellular matrix components. All the subsequent steps have to be carefully executed to avoid contamination or potential infections by media and supplements. So far, most approaches to tissue repair by autologous cells use biopsies from healthy sites on contra lateral tissues such as joint cartilage for articular cartilage repair and nasal septal cartilage for facial plastic reconstruction. The shortfalls of these protocols are obvious: the small number of available cells, the morbidity at the donor site and the limited ability of the harvested cells to proliferate and undergo differentiation. It is not clear whether nasal or auricular chondrocytes could potentially be used for joint repair. Meanwhile, research is increasingly focused on tissue regeneration by relevant precursor or multipotent stem cells. For a successful transfer into clinics, two major goals have to be achieved: (1) a simple and minimal invasive procedure to collect cells from the patient and (2) differentiation of crucial functional properties (e.g. mechanical stability) *in vitro* or *in vivo* within a short time.

Scaffolds for tissue construction

Specially designed biomaterial scaffolds are one of the key components in tissue engineering. Research is focused on developing bioresorbable scaffolds that exhibit optimal physical properties coupled with excellent biocompatibility. Scaffolds act as shape and guidance templates for *in vitro* and *in vivo* tissue development [11]. For cartilage and bone tissues, a suitable scaffold provides initial mechanical stability and supports even cell distribution. Natural polymeric gels, such as hyaluronic acid, collagen, alginate [12] and chitosan, have been used successfully [13] (Fig. 2). These scaffolds permit 3D immobilization of cells and maintain the differentiated phenotype of chondrocytes [14]. However, their mechanical behaviour is insufficient for tissue transplantation and so solid bio-resorbable fibre scaffolds or other porous structures are used to achieve initial biomechanical stability [15]. Synthetic biodegradable poly(α-hydroxy esters) such

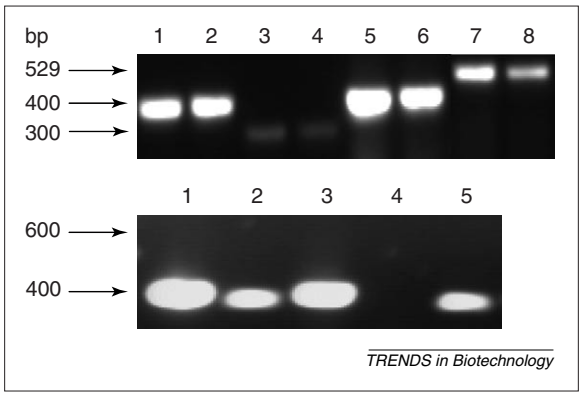

Fig. 2. Expression of various extracellular matrix proteins by chondrocytes growing on hydrogel scaffolds. (a) Gel picture shows the expression of Collagen Type II (COL II), aggrecan and COL IX obtained from two independent experiments. Hypoxanthine phosphoribosyltransferase (HPRT) was used as loading control to compare expression obtained from different experiments. Lane 1, 2 HPRT, Lane 3, 4 aggrecan, Lane 5, 6 COL II, Lane 7, 8 COL IX. Lane 1, 3, 5, 7: Experiment I and Lane 2, 4, 6, 8: Experiment II. (b) Comparative expression of COL I, COL II and COL III. Lane 1, 2, 3, 4 and 5 represents HPRT, COL I, COL II, COL X and COL III, respectively. Note higher expression ($p<0.05$) of COL II in comparison to COL I and III. Expression of COL X could not be detected. (Adapted from [13] and reproduced, with permission, from Cognizant Communication Corporation, NY, USA)

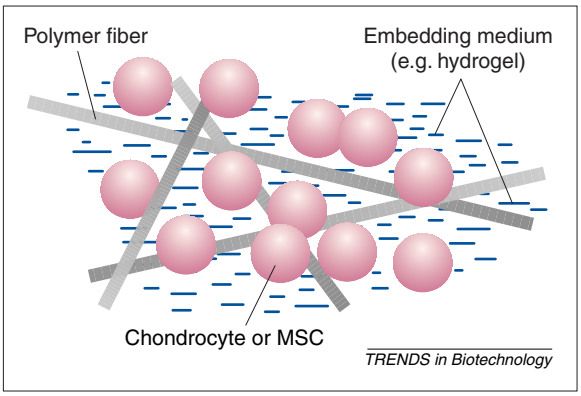

Fig. 3. Schematic drawing showing the strategy of developing tissue engineered cartilage constructs using fibres and embedding substances. Embedding substances offer 3D immobilization and uniform distribution of cells in the fibre mesh. (Figure adapted from [36].)

as polylactic acid (PLLA), polyglycolic acid (PGA) and copolymer PLGA [16] have been used extensively in this context. Both types of materials increase proteoglycan synthesis compared with collagen scaffolds [17]. Injectable *in situ* crosslinkable polymeric preparations that entrap cells have been designed [18] and techniques that combine the advantages of both porous fibre structures and gels are being explored as suitable alternatives to either gels or fibre scaffolds [19] (Fig. 3). Research is also focused on developing 'smart scaffolds' that incorporate inflammatory inhibitors or antibiotics. Slow and controlled release of these bioactive molecules provides sufficient time to the new cartilage to adapt and mature in a 'hostile' *in vivo* situation or to prevent early infection after surgery.

Bioreactors

Although our understanding of cell biology has increased enormously in recent years, the methods of handling *in vitro* cultures of human cells have hardly changed. As demonstrated recently, the ability of conventional monolayer cultures to generate highly differentiated structures is limited because cells are cultured on an inappropriate substrate owing to lack of the requisite characteristic extracellular matrix environment. Moreover, metabolic conditions in the culture medium fluctuate and high density, long-term cultures are always at a risk of contamination [20].

To address the aforementioned problems artificial tissue constructs are cultured in rotating bioreactor vessels or perfusion culture systems (Fig. 4). Experimental data using bovine chondrocytes suggests that hydrodynamic conditions in tissue-culture bioreactors induces glycosaminoglycans (GAGs) and collagen respectively 75% and 39% and mechanical properties such as equilibrium modulus, dynamic stiffness, hydraulic permeability and streaming potential of ~20% of values measured in native cartilage. Cultivation period of 7 months in rotating bioreactors resulted in the wet weight fraction of GAGs and equilibrium modulus equivalent or exceeding the corresponding values measured from freshly explanted native cartilage [21].

Perfusion culture permits the high nutrient consumption in high-density cell cultures and minimizes the accumulation of acidic degradation products from the polymers [22]. Perfusion culture not only optimizes the delivery of components of the culture medium to cells but also stabilizes the secreted levels of autocrine factors such as morphogenetic signals and does not allow build up of synthesized paracrine factors. In addition, by using gradient chambers, a concentration gradient of differentiating factors across an artificial tissue similar to that found during embryonic development can be provided to facilitate specific tissue development.

Use of precursor and mesenchymal stem cells

A fundamental tissue engineering approach to tissue repair is the delivery and integration of functionally active cells, within an appropriate carrier system with respect to cartilage to restore pathologically altered architecture and function. The availability of autologous differentiated cells, such as chondrocytes, is restricted and their functional state does not favour regeneration. Consequently, interest has switched to the use of uncommitted mesenchymal progenitor cells. Recent evidence indicates that even differentiated tissues contain populations of undifferentiated multipotent cells that have the capacity to regenerate tissue after trauma, disease or ageing [23].

Pluripotent embryonic stem cells, successfully cultured from human foetal tissue can differentiate into virtually every tissue and organ of the body. For this reason they are viewed as having an unlimited capacity for cell and tissue replacement therapy [24]. Attempts are ongoing to clone human embryos to derive autologous multipotent cells that can give rise to arrays of cell types in the body [25]. However, the ethical

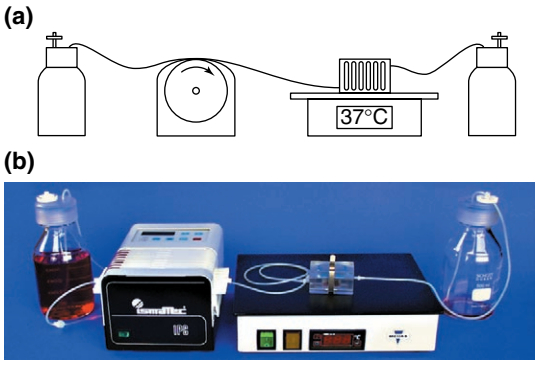

Fig. 4. Perfusion culture unit used to develop tissue engineered tissue constructs. (a) Schematic diagram (b) perfusion culture setup (Minucells and Minutissue). Perfusion culture permits the high nutrient consumption in high-density cultures and minimizes the accumulation of acidic degradation products from the polymers. More defined and stable culture conditions for tissue-engineered tissues are achieved using this apparatus compared with traditional methods.

and social issues associated with the use of these approaches have to be resolved before clinical therapy is possible. Currently, wide attention has been focused on adult mesenchymal stem cells (MSCs) from the bone marrow, in which they reside as supportive cells for haematopoiesis and possibly as a reservoir and regeneration pool for various mesenchymal tissues [26].

The MSCs are characterized by their ability to proliferate in culture and by their properties to differentiate into multiple mesenchymal lineages under defined culture conditions [27] (Fig. 5). They express a defined set of surface markers. The frequency of appearance of MSCs in bone marrow varies between $1{:}10^4$ and $1{:}10^6$ and decreases with the age of the donor. Studies have shown that these cells express the surface molecules CD44, CD71, CD90, CD106, CD120a, CD124 but are negative for haematopoietic lineage markers such as CD14, CD34, CD45 [28]. Besides the expression of surface molecules there is the appearance of a distinct pattern of secreted cytokines, which include IL-6, IL-11, Leukemia inhibitory factor (LIF), Granulocyte macrophage colony stimulating factor (GM-CSF) [29]. Identification of MSCs *in situ* has been an uphill task, partly owing to the relative scarcity of specific molecular markers. Recently, several new antibodies against surface proteins of human MSCs have been established, one of these, SB-10, is directed against surface proteins of uncommitted mesenchymal progenitor cells.

Several experimental studies have evaluated the potential of MSCs to generate cartilage when embedded in an appropriate carrier structures. In principle, transplantation of mesenchymal progenitor cells would ease or possibly correct genetic disorders of bone, cartilage and muscle similar to that reported in a study of children suffering from osteogenesis imperfecta [30]. Moreover, the functional capabilities of MSCs make them likely candidates for gene therapy strategies designed to enhance the process of tissue regeneration and repair and to deliver essential biological signals to restore and maintain tissue homeostasis [31]. Genetic disorders that could be amenable to MSC therapy include degenerative disorders, such as OA and osteoporosis, and inflammatory diseases, such as RA.

Challenges for tissue engineered cartilage

Mechanical stability and graft fixation

The goal of *in vitro* engineering human cartilage is to achieve mechanical properties comparable to those of native cartilage. Studies on *in vitro* engineered cartilage using bovine chondrocytes show that prolonged culture times in the presence of hyaluronic acid enhance the development of tissue that shows impressive mechanical stability and vitality [16]. *In vivo* tissue-engineered cartilage achieves pressure

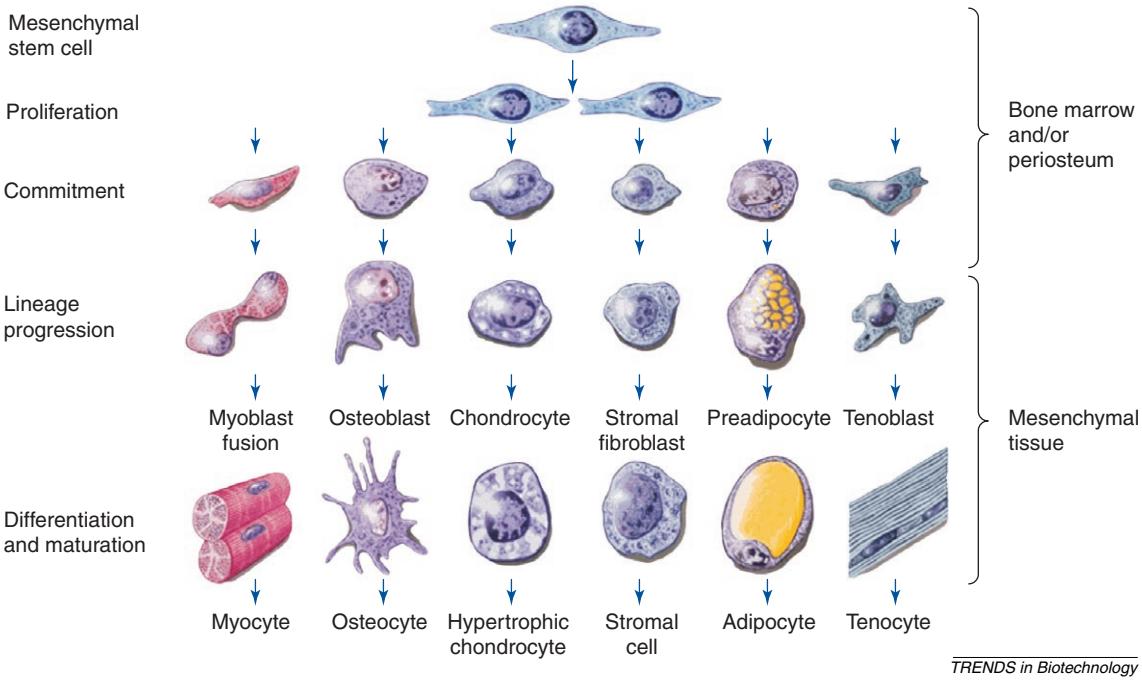

Fig. 5. The schematic drawing depicting mesengenic process. Mesenchymal stem cells (MSCs) differentiate in to a variety of tissues including muscle, bone, cartilage, marrow, fat and ligaments etc. Proliferating MSCs enter a specific lineage following their commitment to that pathway. Commitment of MSCs to a peculiar pathway involves interplay of various morphogens. Lineage committed cells progress to lineage progression through several transitory stages. These cells then undergo a stage of differentiation, which involves cessation of proliferation and biosynthesis of tissue specific proteins and extracellular matrix. Finally, differentiated cells undergo maturation in which they acquire the ability to function in tissue homeostasis. There is a constant turnover of cells taking place in the tissues where dead cells are replaced by newly differentiated cells arising from the continuous transition down the lineage pathway.

resistance and stiffness values comparable to those of native human septal cartilage. However, as yet it is not clear to what extent cartilage transplants in joint defects develop appropriate mechanical properties. The histology of regenerated cartilage in joints usually reveals clear differences between native and transplanted cartilage: the distribution of cells appears somewhat random, lacking typical column formation, and there are no indications of typical collagen architecture, such as arcades. It is also questionable whether there is enough water binding capacity to provide the tissue with appropriate hydroelastic properties.

Another major problem, which has to be solved, is the fixation of the cartilage transplant to the subchondral bone in the joint. In theory, the artificially grown cartilage layers could be attached directly to the defect joint surface using fibrin glue, or it could be fixed using resorbable pins. Currently, preliminary studies of patients with arthroscopically applied *in vitro* engineered cartilage flaps are ongoing (Erggelet, C., Sittinger, M., unpublished observations). Another strategy that has gained attention is to develop an osseo-integrating interface to the cartilage implant using either a calcium carbonate or hydroxyapatite–tricalcium phosphate (HA–TCP) scaffold or an engineered osteoblast layer [32]. The ultimate aim of these studies is to achieve a permanent, solid connection between cartilage and bone tissue.

Immunological aspects

Two major immunological risks are linked to the use of engineered cartilage transplants. First, even when the amount of the biomaterial is reduced, foreign body giant cells or granulocytes are attracted by the scaffolds or certain embedding materials and invade the hybrid tissues. Second, because the engineered tissue is not fully mature, cell surface or matrix protein epitopes are exposed. These epitopes are usually masked from the immune system and might therefore be recognized as 'foreign'. Patients who have received cartilage transplants, showed humoral reactions against type IX and XI collagens, which are associated with collagen, type II fibrils [33]. Clearly, even though tissue engineering is usually regarded as an autologous therapy, a major clinical breakthrough would be to solve the immunological problems associated with treatment.

Applications

Gene therapy and in vivo *applications of tissue engineered cartilage*

Tissue engineering offers the lucrative possibility to treat inflammatory joint disease (IJD). IJD is aggravated by cytokines such as TNF-α and IL-1 [2,3] and therefore transplanted tissue-engineered cartilage would face the risk of cellular damage owing to cytokine exposure. As a result, to regenerate native cartilage and protect tissue-engineered cartilage transplants from additional destruction during inflammatory and destructive joint diseases, cartilage engineering can be combined with approaches to augment the regenerative potential and the matrix production of the transplant. The regenerative and anti-inflammatory potency of the transplant can be maximized by using genetic engineering techniques. For example, it is possible to transfer genes of the TGF-β-superfamily (BMPs) to transplant cells [34]. *Ex vivo* or *in vitro* gene therapy has the particular advantages that: a defined population of cells is genetically modulated; the effects of the gene therapy can be tested to control for a devastating outcome, for example by accidental tumourigenesis and the dosage for optimal differentiation and protection can be controlled.

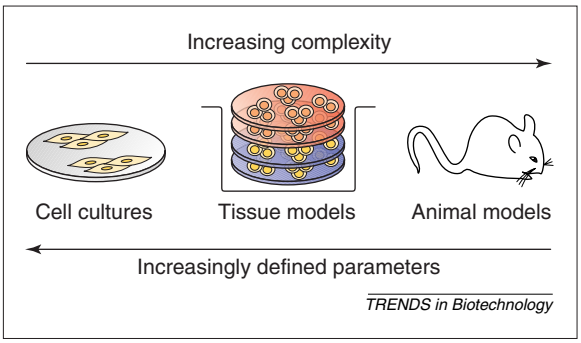

Fig. 6. Potential of tissue models to replace animal models. 3D tissue systems provide a new experimental paradigm to investigate cell function under physiological and pathophysiological conditions, in this way the tissue system provides new and complementary information for animal experiments.

Tissue engineered cartilage constructs are likely to have an impact on treatment modalities offered in a variety of medical disciplines including urology [12], otolaryngology [15,13] and orthopedics. Custom-shaped, autologous grafts for clinical reconstruction of a cartilage defects such as congenital microtia [15] and tracheal agenesis or atresia could be visualized.

Potential in vitro *applications*

These 3D tissue systems provide a new experimental paradigm to investigate cell function under physiological and pathophysiological conditions, in this way the tissue system provides new and complementary information for animal experiments (Fig. 6 and Table 2). *In vitro* models and assay systems are currently used to study cellular differentiation and by influence of various biomolecules, on cellular morphology and behaviour. A new approach is the overexpression or deletion of defined genes (gain or loss of function) in inducible systems. 3D tissue models have the advantage that they can be used to study induction processes in developmental biology, a domain currently dominated by the use of animal models.

It is now known that the local microenvironment can have a profound effect on cell differentiation and function. For this reason newly developed culture models have been designed to investigate the interactions between cell populations and specific extracellular matrix components. An advantage of this approach is that a defined set of cells, which can be of autologous origin, can be isolated from a specific tissue

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Table 2. *In vitro* applications of 3D tissue models

Application	Example
In vitro assay	Test system for drugs, cytokines, morphogenetic factors and enzyme inhibitors
Morphogenesis model	Induction of proliferation and differentiation in an interactive 3D culture
Establishment of tissue transplants	Combination with scaffolds as supportive structures
Angiogenesis model	Endothelial cells interacting with tumor cells, inflammatory cells and so on
Cell migration	Migration of mononuclear cells, fibroblasts and so on in an extracellular matrix, chemotaxis, cell adhesion, homing and infiltration
Immunological studies	Interaction of T cells with macrophages, antigen presenting cells and fibroblasts in context of the extracellular matrix
Genetically altered cells	Transfection of mesechymal stem cells for the expression of morphogens and interaction with resident cells

and genetically altered, the phenotype and function of these cells can be investigated under reproducible and specific culture conditions. The experimental strategy can thereby focus on various aspects of cell dynamics, such as structural and functional changes, cellular activation, migration, infiltration or degradation of the pericellular matrix, synthesis of specific proteins or apoptosis. The recently designed bioreactor systems allow the establishment of separate cellular compartments depending on the experimental setting (e.g. serial culture, mixed culture and direct cell-cell contact). As a consequence, *in vitro* tests for drugs or bioactive molecules can be developed [35].

Conclusions

The key to successful repair and regeneration of cartilage is to provide the repair site with sufficient chondrogenic cells in a suitable delivery vehicle to ensure maximal differentiation and deposition of right extracellular matrix. New optimized culture methodologies and bioreactors that provide appropriate mechanical and other guidance clues must be engineered to ensure the successful function of engineered tissue. As we gain more and more information about the identity of all of the morphogens for chondrocyte differentiation it might be possible to orchestrate massive cartilage regeneration by clever combination of smart 3D scaffolds and such morphogenic factors. The management of cells both *in situ* and *ex vivo*, will be crucial to the success of such tissue engineering efforts.

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