Review

Chondrogenesis and cartilage tissue engineering: the longer road to technology development

Nastaran Mahmoudifar[1] and Pauline M. Doran[2]

[1] School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia
[2] School of Biological Sciences and Department of Chemical Engineering, Monash University, Melbourne, VIC 3800, Australia

Joint injury and disease are painful and debilitating conditions affecting a substantial proportion of the population. The idea that damaged cartilage in articulating joints might be replaced seamlessly with tissue-engineered cartilage is of obvious commercial interest because the market for such treatments is large. Recently, a wealth of new information about the complex biology of chondrogenesis and cartilage has emerged from stem cell research, including increasing evidence of the role of physical stimuli in directing differentiation. The challenge for the next generation of tissue engineers is to identify the key elements in this new body of knowledge that can be applied to overcome current limitations affecting cartilage synthesis *in vitro*. Here we review the status of cartilage tissue engineering and examine the contribution of stem cell research to technology development for cartilage production.

Cartilage tissue engineering: prospects and challenges

Articular cartilage has little capacity for self-repair but a relatively high incidence of damage and deterioration from common trauma such as sports injury and diseases such as osteoarthritis. Surgical procedures such as autologous chondrocyte implantation for cell-based repair of small chondral lesions, and subchondral bone drilling or microfracture to activate cartilage synthesis by progenitor cells, are practiced clinically. However, despite providing temporary relief from the symptoms of pain and swelling associated with cartilage failure, these approaches give mixed results [1,2]. In many cases, the outcome is the formation of fibrous repair tissue or fibrocartilage that does not possess the full load-bearing properties and durability of healthy articular cartilage. Ultimately, total replacement of the joint is often required.

Tissue engineering offers an alternative solution using cells to produce cartilage tissues outside of the body that are suitable for implantation and repair of large chondral defects. The extent of *in vitro* differentiation and development required for tissue engineering applications remains unclear and debatable: isolated cells, cells embedded in a scaffold or matrix, partially formed tissues, and mature, fully formed tissues and organs are some of the options. The ultimate goal requiring deployment of the widest range of scientific and technical skills is the generation of fully functional tissues using cell culture systems. For cartilage, this means the production of matrix that mimics as closely as possible the biochemical and mechanical properties of native articular cartilage (Box 1).

Because articular cartilage is a thin, avascular and aneural tissue requiring only one type of cell for synthesis, it has been considered a relatively easy target for tissue engineering. Along with skin, cartilage was one of the first tissues to be investigated for commercial production. However, despite the efforts of many researchers over the last 15–20 years, production of functional cartilage constructs suitable for either clinical or nonclinical applications remains an elusive goal. Although a wide variety of cell types, differentiation factors, scaffolds, bioreactors and culture conditions has been tested to promote cartilage synthesis, effective and reliable culture strategies yielding tissues with properties matching those of native cartilage have not been developed. Accordingly, the task of generating functional cartilage *in vitro* remains just as challenging as ever.

This review examines the current status of cartilage tissue engineering and its prospects for the future. We assess the progress made in terms of the quality of the tissues produced thus far using differentiated chondrocytes and stem cells. We discuss the practical implications of new insights into differentiation afforded by stem cell research, and identify areas that present the greatest hurdles for improving cartilage production. Despite rapid developments in the field, substantial problems remain for cartilage tissue engineering.

Cartilage from differentiated chondrocytes

Cartilage tissue engineering using chondrocytes has been studied extensively and many three-dimensional scaffold and bioreactor culture systems have been developed. Chondrocytes are isolated from cartilage tissue, but useful quantities of healthy human cartilage from load-bearing joints are difficult to source because of the high risk of joint injury at the donor site. Consequently, most tissue engineering studies using differentiated chondrocytes have employed animal models or human fetal chondrocytes rather than human adult cells. Representative data for the biochemical composition of tissue-engineered cartilage

Corresponding author: Doran, P.M. (pauline.doran@monash.edu).

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Box 1. Properties of human adult articular cartilage

The principal constituents of articular cartilage that give this tissue its remarkable mechanical and load-bearing properties are collagen type II for tensile strength and glycosaminoglycans (GAGs) for resilience, compressive stiffness and load distribution. Chondrocytes, the cells responsible for synthesizing and maintaining cartilage, comprise only approximately 1% of the mature tissue volume. Water accounts for approximately 60–85% of the tissue weight. Within the dry solid matrix, 50–75% w/w is collagen and 15–30% w/w is proteoglycan [74,75]. Several different collagen types are found in articular cartilage; however, 90–95% of the collagen present is in the form of collagen type II fibrils (Figure 1a). The primary proteoglycan in articular cartilage is aggrecan, which consists of a core protein with many unbranched GAG side chains. The strong negative charge associated with GAG and the resulting tendency of the tissue to imbibe water give cartilage its compressive stiffness. Values of the tensile Young's modulus for healthy human cartilage fall within the range 5–25 MPa depending on the joint and depth within the tissue. The compressive aggregate modulus ranges from 0.08 to 2 MPa, with an average of approximately 800 kPa. During normal joint movement, the total compressive strain is 15–20% [75].

constructs produced using human chondrocytes are shown in Table 1. These data represent neither the highest nor lowest values reported, but show trends that are typical of results from a large number of investigations using a variety of cell sources and culture systems. For comparison, the biochemical compositions of native human fetal and adult articular cartilage are also listed.

Whereas glycosaminoglycan (GAG) levels in chondrocyte-produced constructs often approach or exceed those in native adult articular cartilage, the most important limitation for cartilage synthesis using chondrocytes is inadequate accumulation of collagen [3,4] (Table 1). Despite its significance as a key marker of chondrogenesis, collagen type II has not been measured quantitatively in many cartilage engineering studies. Because collagen type I is often expressed at undesirably high levels, measurements of total collagen reveal neither the differentiation status of the cells nor whether cartilage-like tissue is being produced. Typically, collagen type II concentrations in cultured constructs remain substantially lower than those in native tissues, even though values for collagen type II as a percentage of total collagen may be similar (Table 1). This suggests that chondrocytes do not retain a stable chondrocytic phenotype or ability to produce cartilage tissue *in vitro*, even when cultured in three-dimensional scaffolds, so that matrix synthesis and deposition do not reach mature levels. Tissue-engineered constructs have been generated with compression moduli similar to that of native cartilage [5,6], which reflects the accumulation of GAG at concentrations roughly matching those in native tissues. However, because collagen is responsible for the tensile strength of cartilage, low collagen levels mean that the overall mechanical properties of engineered constructs remain inferior [7,8].

Relative to adult cartilage, tissue-engineered constructs usually exhibit higher cellularity, higher GAG and water contents, and lower total collagen and collagen type II contents. These properties are broadly similar to those of fetal cartilage compared with adult cartilage (Table 1). Although similarity to fetal cartilage may be disappointing if the goal is to produce cartilage with the functional properties of adult tissue, according to the biomimetic or developmental engineering approach to tissue engineering [9–11], constructs *in vitro* may need to progress through all developmental stages to achieve an authentic outcome. Synthesis of tissues with properties similar to fetal cartilage could therefore be an important

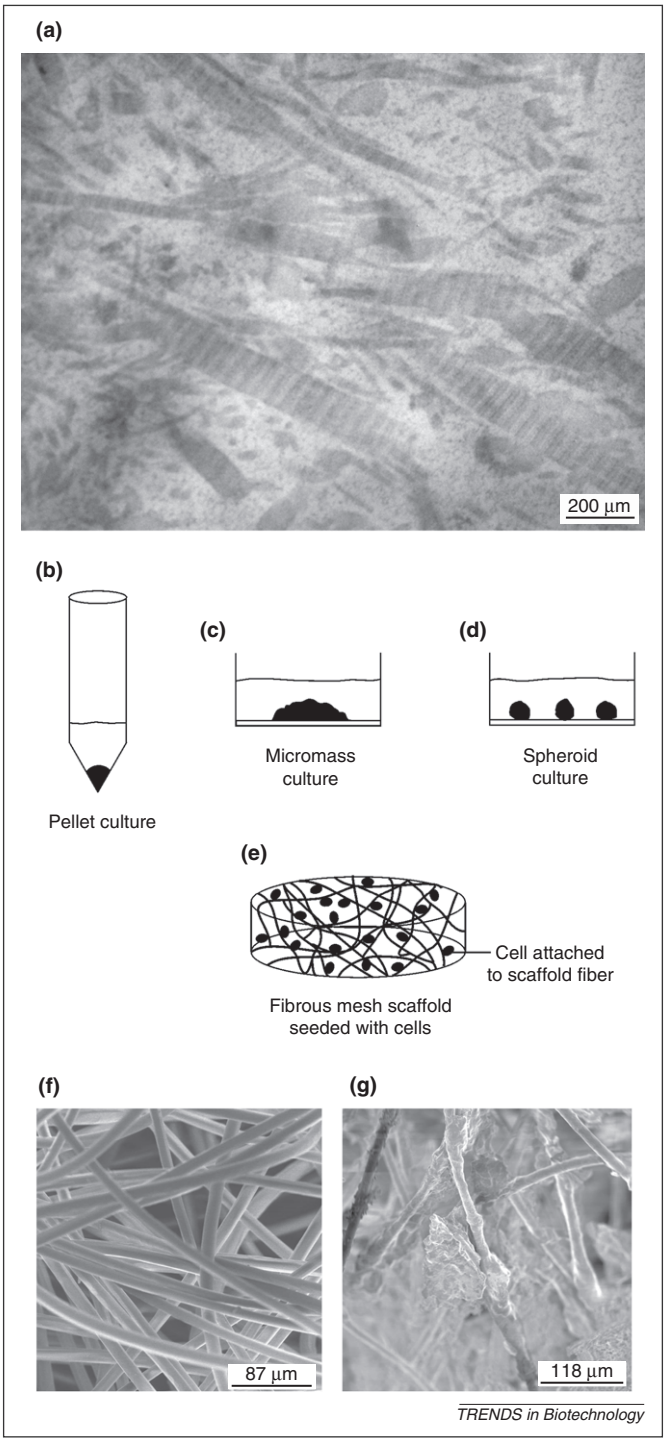

Figure 1. **(a)** Transmission electron micrograph of human adult articular cartilage showing large banded fibrils of collagen type II. The cartilage matrix also contains proteoglycans embedded within the collagen network. (b–d) Methods for scaffold-free three-dimensional stem cell culture: **(b)** pellet culture; **(c)** micromass culture; and **(d)** spheroid culture. **(e)** Schematic of a porous fibrous scaffold, such as PGA polymer mesh, seeded with cells. (f,g) Scanning electron micrographs: **(f)** PGA fibers within an unseeded PGA mesh scaffold; and **(g)** PGA fibers with attached cells after 3 days of cell seeding.

Table 1. Properties of tissue-engineered cartilage constructs and human fetal and adult articular cartilage[a]

Culture system or cartilage source	Tissue property						Ref.
	Cell concentration (cells g^{-1} dw$\times10^{-6}$)	Water content (% w/w)	GAG concentration (% dw)	Total collagen concentration (% dw)	Collagen type II concentration (% dw)	Collagen type II as a percentage of total collagen (%)	
Tissue-engineered cartilage using human chondrocytes after 5 weeks of culture	340±13	90±0.3	26±2.1	11±3.4	8.5±1.8	88±11	[70]
Tissue-engineered cartilage using human adipose-derived stem cells after 5 weeks of culture	340±24	84±1	2.5±0.076	14±2.0	0.22±0.054	1.6±0.15	[31]
Human fetal articular cartilage	1350±43	89±0.4	42±4.1	19±1.5	17±1.6	77±8.4	[71]
Human adult articular cartilage	40±10	79±0.3	17±0.51	54±0.39	47±6.3	86±11	[68]

[a]Results are mean±standard error for triplicate bioreactor cultures or ex vivo cartilage samples. dw, dry weight.

milestone on the way to mature cartilage, provided that methods to induce further cartilage maturation can also be developed.

Cartilage from mesenchymal stem cells

Ongoing ethical issues and immunorejection problems using embryonic stem cells and unresolved safety concerns about the tumorigenicity of embryonic and induced pluripotent stem cells [12] mean that tissue-derived mesenchymal stem cells represent the most practical stem cell type for cartilage tissue engineering. In particular, adipose-derived stem cells are an attractive resource for clinical applications. Adipose tissue is easy to access and in plentiful supply in most patients; mesenchymal stem cells are also more abundant in lipoaspirates than in bone marrow [13]. The relative ease with which autologous mesenchymal stem cells can be obtained is an important advantage compared with differentiated chondrocytes for engineering of human cartilage.

Application of stem cells for cartilage production requires transformation of the cells into chondrocytes capable of producing functional cartilage matrix. Chondrogenesis, the key to success using stem cells, is currently a major stumbling block. If problems with differentiation and phenotype stability hindered the success of tissue engineering using chondrocytes, these difficulties are magnified, not solved, using stem cells. Several comparative studies have shown that the quality of cartilage produced using mesenchymal stem cells is substantially lower than that obtained using chondrocytes [14–18]. Representative data illustrating this comparison for human cartilage are presented in Table 1.

How complicated can differentiation be?

A wealth of new information about the complex biology of differentiation is emerging from current research into the molecular biology of stem cells. Rather than single or a few key driving signals being responsible for differentiation, cascades of biochemical interactions involving extensive regulatory networks that overlap and crosstalk between different tissue types are being elucidated. Dozens or perhaps hundreds of different genes and effector molecules have been shown to influence the course of chondrogenesis (Table 2) and new chondrogenesis-related genes and

Table 2. Some biomolecules involved in chondrogenesis *in vitro* [72,73]

Molecule	Effect
Actin	Transduces the chondroinhibitory effects of cell–integrin adhesion; disruption of the actin cytoskeleton stimulates re-expression of cartilage genes in dedifferentiated chondrocytes
Activator protein-1 (AP1) transcription factor	Negative regulator of chondrogenesis; upregulated by WNT7A to inhibit chondrogenesis
Activin	Upregulates expression of NCAM during condensation; increases tenascin accumulation in mesenchymal aggregates and differentiated nodules
Bone morphogenetic proteins (BMP2, -4, -6, -7, -9, -12, -13)	Regulate proliferation and maturation of chondrocytes; upregulate N-cadherin expression to promote cell–cell interactions; enhance proteoglycan and collagen type II accumulation; promote *SOX9* expression; enhance stimulatory effects of TGF-β
β-Catenin	Mediates WNT3A signaling; WNT/β-catenin signaling promotes hypertrophy independent of IHH signaling; SOX9 inhibits activation of β-catenin-responsive promoters and stimulates β-catenin degradation
Connective tissue growth factor (CTGF/CCN2)	Expression regulated by TGF-β/SMAD signaling, VEGF, retinoic acid, RAC1 signaling, changes in actin cytoskeleton, and mechanical stimuli; expression leads to changes in focal adhesion complexes, actin networks, and cell morphology
CREB binding protein (CBP)	Transcriptional cofactor able to bind SOX proteins
C-1-1 transcription factor	Overexpression inhibits hypertrophic differentiation
Dexamethasone	Enhances chondrogenesis; enhances stimulatory effects of TGF-β3
Epidermal growth factor (EGF)	Inhibits condensation and proteoglycan accumulation
Fibroblast growth factors (FGF2, -4, -8)	Inhibit or enhance chondrogenesis depending on cell type and conditions; mediate the balance between SOX9 and RUNX2

Table 2 (*Continued*)

Molecule	Effect
Growth and differentiation factor-5 (GDF5)	Enhances chondrogenesis
Histone deacetylases (HDAC1, -4)	HDAC1 knockdown reduces cartilage development; HDAC4 inhibits chondrocyte hypertrophy and mineralization; inhibition of HDAC blocks cartilage matrix production and redifferentiation of dedifferentiated chondrocytes and enhances WTN5A expression
Homeodomain (HOX) transcription factors (BARX2, NKX3-2, MSX1, MSX2, PAX1, PAX9)	BARX2 stimulates chondrogenesis and cartilage synthesis; NKX3-2 stimulates chondrogenesis under the control of BMP-dependent association with SMAD1 and SMAD4; overexpression of PAX1 induces NKX3-2 expression and chondrogenesis; PAX1 and PAX9 interact with and transactivate the *NKX3-2* promoter; MSX2 represses chondrogenic differentiation
Indian hedgehog (IHH)	Major regulator of chondrocyte hypertrophy; activates *PTHRP* expression as part of a negative feedback loop
Insulin-like growth factor-1 (IGF1)	Enhances accumulation of cartilage matrix; promotes aggrecan and collagen type II expression in combination with TGF-β1 or TGF-β2 and/or FGF2
β1-Integrins	Cell surface receptors mediate cell–matrix interactions; essential for cell signaling, communication and regulation of chondrocyte-specific gene expression; function as mechanoreceptors in the chondrocyte mechanotransduction pathway
Lymphocyte enhancer binding factor-1 (LEF1) transcription factor	Positive regulator of chondrogenesis; transduces chondrostimulatory WNT3A signaling
Leukemia/lymphoma-related factor (LRF)	Overexpression depresses BMP2-induced chondrogenesis
Matrix metalloproteinases (MMP1, -2, -13)	MMP1 and MMP2 are upregulated during early chondrogenesis to reduce protein content of matrix; MMP13 degrades collagen type II in late-stage chondrogenesis
Mitogen-activated protein kinase (MAPK) signaling pathways (p38 pathway)	Regulated by mechanical stimuli
Neural cadherin (N-cadherin)	Cell adhesion molecule required for condensation; subsequent downregulation required for differentiation; activation required for expression of SOX9, SOX5, SOX6, aggrecan, and collagen type II; expression regulated by TGF-β, BMP2, different WNTs, and RAC1 signaling
Neural cell adhesion molecule (NCAM)	Positive regulator of condensation
Noggin	BMP antagonist
Paired box-containing gene (PAX) transcription factors	PAX1 can substitute for SHH to initiate chondrogenesis; SHH and noggin are upstream regulators of PAX1
Parathyroid hormone-related peptide (PTHRP)	Major regulator of chondrocyte hypertrophy; acts together with IHH as part of a negative feedback loop; regulates SOX9; PTHRP signaling is inhibited by WNT/β-catenin signaling
Perlecan	Induces cell aggregation, condensation and chondrogenesis; can bind to cartilage matrix components, FGFs, and BMPs
Prostaglandin-E2 (PGE2)	Inhibition of PGE2 synthesis reduces chondrogenesis
Protein kinase A (PKA)	Positive regulator of chondrogenesis; BMP2 is an upstream activator of PKA; phosphorylation of SOX9 by PKA stimulates SOX9 transcriptional activity
Protein kinase C (PKC) family (PKCα, PKCγ, PKCε)	Promote chondrogenesis; PKCα may mediate effects of changes in the actin cytoskeleton during chondrogenesis
Retinoic acid	Inhibits or enhances cartilage matrix formation
Rho family of GTPases (RHOA, RAC1, CDC24)	RHOA suppresses chondrogenesis via ROCK1/ROCK2-dependent mechanisms; overexpression of RHOA induces actin filament organization and stress fibers, increases cell proliferation, decreases cartilage matrix accumulation and suppresses hypertrophy; RHOA-mediated modulation of actin polymerization regulates *SOX9* transcription; Rho family members control the actin cytoskeleton and regulate CTGF/CCN2; overexpression of RAC1 and CDC24 induces hypertrophy; WNT3A signaling activates RAC1
Rho kinases (ROCK1, ROCK2)	Downstream effectors of RHOA; activate *SOX9* in response to mechanical compression and TGF-β; inhibition of ROCK1/2 enhances cartilage matrix accumulation, cell rounding and reorganization of the actin cytoskeleton characteristic of chondrogenesis
Runt-related transcription factor 2 (RUNX2)	Osteogenic transcription factor; balance with SOX9 determines osteo- or chondro-differentiation
SMAD transcription factors (SMAD1, -2, -3, -4, -5, -7, -8)	Promotion of chondrogenesis by individual TGF-β and BMP growth factors involves activation of unique combinations of downstream SMADs; BMP-induced SMAD1 and interactions between SMAD1 and RUNX2 regulate hypertrophic transformation
Sonic hedgehog (SHH) signaling protein	Promotes chondrogenesis; has synergistic effect with FGF8
SP1 and SP3 transcription factors	Repression of collagen type II expression by SP1 and SP3 requires HDAC1
SRY-type high mobility group box (HMG-box) DNA-binding domain (SOX) transcription factors	*SOX9* expression is required for condensation and chondrocytic differentiation; all chondro- and osteo-progenitors arise from *SOX9*-expressing cells; *SOX9* expression is regulated by the TGF-β, FGF, BMP and WNT families; SOX9 is responsible for expression of *SOX5* and *SOX6* and upregulation of collagen type II; L-SOX5 and SOX6 are required for aggrecan and collagen type IX expression; SOX9 inhibits chondrocyte hypertrophy and regulates PTHRP
Syndecan-3	Overexpression blocks BMP2-stimulated SMAD phosphorylation and chondrogenesis; affects activity of tenascin
Tenascin-C	Promotes condensation
TIAM1	Effector of RAC1
Transforming growth factor-β (TGF-β1, -β2, -β3)	Upregulates fibronectin expression during condensation; induces *SOX9* expression; stimulates expression of aggrecan, collagen type II, N-cadherin, NCAM, collagen type XI, fibronectin, tenascin, and decorin

Table 2 (*Continued*)

Molecule	Effect
Vascular endothelial growth factor (VEGF)	Expression upregulated during cartilage hypertrophy
Versican	Enhances condensation; necessary for chondrogenic gene expression
Vimentin	Cytoskeletal protein that acts as a positive regulator of chondrogenesis; knockdown inhibits *SOX9* expression and accumulation of aggrecan and collagen type II
Wingless- and int-related protein (WNT) signaling molecules (WNT1, -3a, -5a, -7)	Effect of WNTs is time- and level-dependent; at low levels, WNTs promote chondroprogenitor differentiation through modulation of *SOX9* expression; at high levels, WNTs promote chondrocyte hypertrophy; WNT5A promotes early chondrogenesis but inhibits terminal differentiation; WNT5 knockdown reduces the inhibitory effect on cartilage production of HDAC inhibition; WNT7 expression inhibits chondrogenesis by blocking the transition from condensation to differentiation; WNT signaling mediated by β-catenin promotes hypertrophy and chondrocyte maturation independent of IHH and PTHRP signaling; WNT/β-catenin signaling antagonizes PTHRP signaling and inhibits chondrocyte lineage determination; WNTs regulate expression of N-cadherin and mediate the balance between SOX9 and RUNX2

proteins continue to be identified using genomic and proteomic techniques [19,20]. There is a strong temporal or scheduling element in chondrogenesis as specific genes are upregulated or downregulated transiently, depending on whether the process is in its initial, middle or final stages [21–23]. The control pathways being revealed are so complex and interconnected that a systems biology approach based on mathematics is becoming not only desirable but necessary to sift through all the data. Biological network analysis is in its infancy; however, the hope is that mathematical and statistical methods will be able to distil the large amounts of information being generated, so that key control points, principal components and major interdependencies that may not be intuitive or readily apparent from the raw empirical data might be identified.

Although some elements of chondrogenesis have been achieved using mesenchymal stem cells, significant problems remain. The persistence of collagen type I expression [22,24,25] suggests that mechanically inferior fibrocartilage is produced. Induction of hypertrophy markers such as collagen type X, matrix metalloproteinase 13, alkaline phosphatase (ALP) and tissue mineralization [22,26] indicates that the differentiation pathways occurring *in vitro* using typical experimental protocols do not generate a normal or stable chondrogenic phenotype. Hypertrophy has been associated with the development of endochondral-like cartilage and ossification *in vivo* after ectopic transplantation of chondroinduced cells [22]. Genome-wide transcriptional screening of articular chondrocytes and mesenchymal stem cells cultured under chondroinductive conditions showed that over 300 genes were misregulated in mesenchymal stem cells during chondrogenesis [17]. Some genes were never expressed, some were expressed at lower levels, and some were expressed at later time points compared with those in chondrocytes. Moreover, chondroinduced stem cells cease to generate additional cartilage matrix after a certain stage of development *in vitro*, even when extended culture periods are provided [14]. Consequently, the quality of the cartilage produced using mesenchymal stem cells is always lower than that of native articular cartilage [16,18,27] (Table 1).

Although genetic manipulation of stem cells has been employed in several studies to provide the regulatory proteins required for chondrogenesis, this approach has not delivered unequivocal benefits for cartilage production (Box 2). At the present time, recruitment of environmental factors to remedy some of the problems encountered with chondrogenesis seems a more useful practical strategy. Manipulation of cell culture conditions is a more distributed approach to influencing differentiation and has the potential to trigger cascades of molecular responses rather than local networks based on single genes or proteins. For example, a promising finding is that hypertrophic development in mesenchymal stem cells is suppressed when the cells are co-cultured with chondrocytes or chondrocyte-like cells, or if chondroinduction takes place in conditioned medium from chondrocyte culture [28,29]. In other work, surface modification of scaffold polymers has also been successful in selectively inhibiting expression of collagen type X [30].

The neglected physical environment

Cellular environments *in vivo* are characterized by a diversity of cell–cell and cell–matrix interactions, oxygen and small-molecule effects, and mechanical forces. These aspects of culture systems, in addition to the biochemical agents provided in the medium, are now known to regulate cell differentiation. Despite this, many studies of chondrogenesis are conducted using pellet or micromass culture of stem cells, whereby cells are cultivated at high density in small clumps under static conditions (Box 3). These forms of culture mimic most directly the condensation phase of chondrogenesis, but are inadequate for matrix development because they do not provide important physical stimuli and biomechanical cues. Compared with static pellet cultures, cartilage production is greater when stem cells are cultured in three-dimensional scaffolds under dynamic culture conditions [31]. The mechanisms responsible for this are just beginning to be revealed.

Physical stimuli exert a potent influence over lineage commitment in stem cells: different tissue-specific cell types can be induced, including across embryonic germ layers, simply by altering scaffold properties [32]. When cells attach to surfaces, change their shape, move or are impacted by mechanical forces, their internal cytoskeleton is distorted, which generates tension that is sensed by cell surface receptors. Mechanotransduction of these signals plays a central role in regulating the transcription of genes governing cell growth and differentiation [33,34]. Changes in cytoskeletal structure are sufficient to determine whether cells grow, differentiate, switch between different lineages or undergo apoptosis. Scaffold material properties

Box 2. Use of genetic transformation to improve chondrogenesis in stem cells

Genetic manipulation has been widely used as a tool to investigate the biochemical pathways and regulatory networks involved in cell differentiation. As an extension of this approach, gene transfer can also be used for practical tissue engineering [76]. Adenovirus- or nonviral-mediated expression of growth factors, transcription factors and other regulatory proteins offers an intracellular solution to the problem of supporting and controlling chondrogenesis [41,77–80]. Alternatively, application of short hairpin RNA (shRNA) to silence collagen type I expression has been applied to suppress fibrocartilage synthesis by mesenchymal stem cells [80], and this strategy could be extended to other unwanted matrix components. However, undesired side effects of these techniques have been found: for example, *BMP2* and *BMP4* gene transfer and collagen type I silencing led to enhanced expression of collagen type X and other markers of chondrocyte hypertrophy [78,80]. Other issues associated with overexpression of specific differentiation factors include lack of control over duration and timing, and ineffectiveness in the absence of other environmental triggers [41,77,79].

The occurrence of deleterious side effects after relatively simple genetic manipulations in stem cells reflects the complexity of the biochemical processes involved in differentiation. Although much has been learnt in recent years about the genes and regulatory factors that influence chondrogenesis, our understanding of the hierarchy of interactions between these molecules and the biochemical bottlenecks affecting chondrogenesis is far from complete. Manipulation of one or even a few genes may not be sufficient to yield substantial improvements in cartilage tissue production unless other conditions have already been optimized. The molecular control systems associated with differentiation and cell fate determination are robust enough to provide multiple avenues for self-correction to overcome the effects of gene transfer, and are sufficiently complex that the chances of unintended knock-on effects are high.

Virally mediated transgene expression has a record of clinical application for treatment of rheumatoid arthritis [81]. However, even though adenoviral transduction is nonintegrative and transgene expression is transient, concerns about the safety of gene therapy and public and professional acceptance of transgene expression in humans, particularly for treatment of nonlethal conditions, remain significant hurdles affecting the potential of these techniques for tissue engineering.

Does gene expression mean cartilage synthesis?

For practical tissue engineering purposes, chondrogenesis is not defined by gene expression alone: synthesis and accumulation of cartilage matrix are the real goal. Upregulated gene expression for aggrecan and collagen type II does not always translate into increased synthesis or accumulation of these compounds [17,24,31,41,82]. Possible reasons for this include post-transcriptional regulation of protein synthesis, translational deficiencies, lack of protein accumulation after synthesis to form an insoluble matrix, and matrix turnover. In particular, significant translational and post-translational factors control collagen synthesis [83,84]. Accordingly, gene expression can be considered an inadequate and potentially unreliable indictor of chondrogenesis for cartilage production. Because differentiation is profoundly affected by the culture environment, characterization or comparison of different tissue-derived stem cells in terms of their chondrogenic potential based on gene expression profiles is necessarily fraught when only one or a limited range of culture conditions is used. The 'chondrogenic potential' of stem cells is in fact difficult to pin down because it is subject to many strong external influences.

such as stiffness, elasticity and hydrophobicity, and elements of scaffold architecture such as fiber diameter and micro- and nanotopography, regulate differentiation through their effects on cell attachment, morphology, migration and the cytoskeletal network [35–37]. Mechanical stimuli such as tension, compression and hydrostatic forces also have the potential to influence cellular differentiation pathways [38].

To exert control over chondrogenesis through rational design of the physical environment, we need to know what specific biological responses are induced by particular scaffold properties or mechanical and hydrodynamic forces. However, the links between the physical environment, intracellular molecular networks and cell phenotype are not yet completely understood. In principle, the development of gel scaffolds with tunable properties allows the effect of particular material properties to be evaluated through systematic variation while other characteristics of the scaffold remain unaltered. In practice, however, this is difficult to achieve in three-dimensional systems because changes in properties such as stiffness and elasticity are obtained by modifying the polymer concentration or ligand density, which unavoidably affects other scaffold properties such as porosity, diffusion characteristics and biodegradability. An important practical outcome of work in this area so far is recognition that the rigid plastic surfaces of plates and flasks commonly used for stem cell culture are likely to generate inappropriate mechanical signals for differentiation and tissue development, except perhaps for bone, and that softer substrates are generally more suitable [36].

Because externally delivered mechanical forces such as tension and compression play a crucial role in joint development and healing, it is reasonable to expect that these factors also regulate chondrogenesis. As might be expected, the specific conditions applied, such as load intensity, duration and frequency, determine whether mechanical forces are beneficial or not. Direct tension or compression of cell-seeded constructs can have both stimulatory and inhibitory effects on chondrogenesis [39,40]; induction of collagen type X expression has also been reported [41]. There is some evidence that treatments such as compressive loading are not helpful during early chondrogenesis, although they enhance subsequent matrix deposition [42–44]. Accordingly, it may be necessary to allow chondrogenesis to occur *in vitro* before mesenchymal stem cells are implanted into load-bearing environments *in vivo* [42,44].

Control of the physical as well as chemical environment is vital for cartilage production, so bioreactor systems (Box 3) have an important role to play in stem cell culture. Compared with tissue flasks, well plates and Petri dishes, bioreactors offer better control over culture conditions, reduced diffusional limitations for delivery of nutrients and metabolites into tissues, regulation of dissolved oxygen tension and gas exchange, and exertion of mechanical and hydrodynamic forces influencing cell and tissue development. Oxygen has a regulatory function in chondrogenesis [45,46] and cannot be delivered effectively into the interior of even small tissue constructs unless fluid convection is present to supplement diffusion. Hydrodynamic shear forces, such as those associated with fluid flow in bioreactors, have the potential to influence chondrogenesis through induction of *SOX9* and *RUNX2* expression and alterations in cytoskeletal tension [47]. Several different bioreactor configurations have been applied for stem cell culture, and it has been shown that the bioreactor type

Box 3. Scaffolds and bioreactors

Scaffold-free methods for three-dimensional culture of stem cells include pellet, micromass and spheroid cultures. For pellet culture, 1–3×10^5 cells are placed in a conical tube and centrifuged to produce a compact cell pellet or aggregate before addition of a small volume of culture medium (Figure 1b). Alternatively, micromass cultures (Figure 1c) are formed from a droplet (10–50 μl) of high-density cell suspension placed on the surface of a tissue culture or well plate. Once the cells are attached, liquid medium is added. Depending on the culture conditions, cells in micromass culture may form compact spherical aggregates or spheroids of diameter 300–500 μm (Figure 1d).

An alternative to these methods is the use of porous scaffolds to provide a three-dimensional structure to support cell and tissue growth. Scaffolds provide solid surfaces for cell attachment while allowing penetration of fluid currents carrying oxygen and nutrients within the developing matrix. Scaffolds take a wide variety of forms; however, porous nonwoven polymer mesh fabricated from biodegradable materials such as polyglycolic acid (PGA) is a typical example (Figure 1e). PGA fibers of diameter 12–14 μm within an unseeded PGA scaffold and with cells attached after seeding are shown in Figure 1f and 1g, respectively.

Scaffolds seeded with cells are suitable for bioreactor culture. Bioreactors are vessels used to cultivate cells under controlled conditions in a dynamic flow or mixed environment. Bioreactor systems may also include auxiliary equipment for gas exchange, temperature control and/or mechanical stimulation of the cells. Examples of bioreactor configurations suitable for cartilage tissue engineering are illustrated in Figure I. One of the simplest designs is the direct perfusion system, in which recirculating medium is forced to flow through porous cell-seeded scaffolds inserted in the flow path (Figure Ia). In rotating bioreactors, constructs are suspended freely in medium located in the annulus between two concentric cylinders (Figure Ib). Mixing occurs as the reactor rotates around its central axis and the rotational flow field interacts with the constructs as they tend to settle owing to gravity. In spinner or stirred flask systems, fluid flow is generated using a magnetic stirrer and the constructs are immobilized, for example on needles fixed within the mouth of the flask, to avoid damage to the tissues from direct contact with the stirrer bar (Figure Ic).

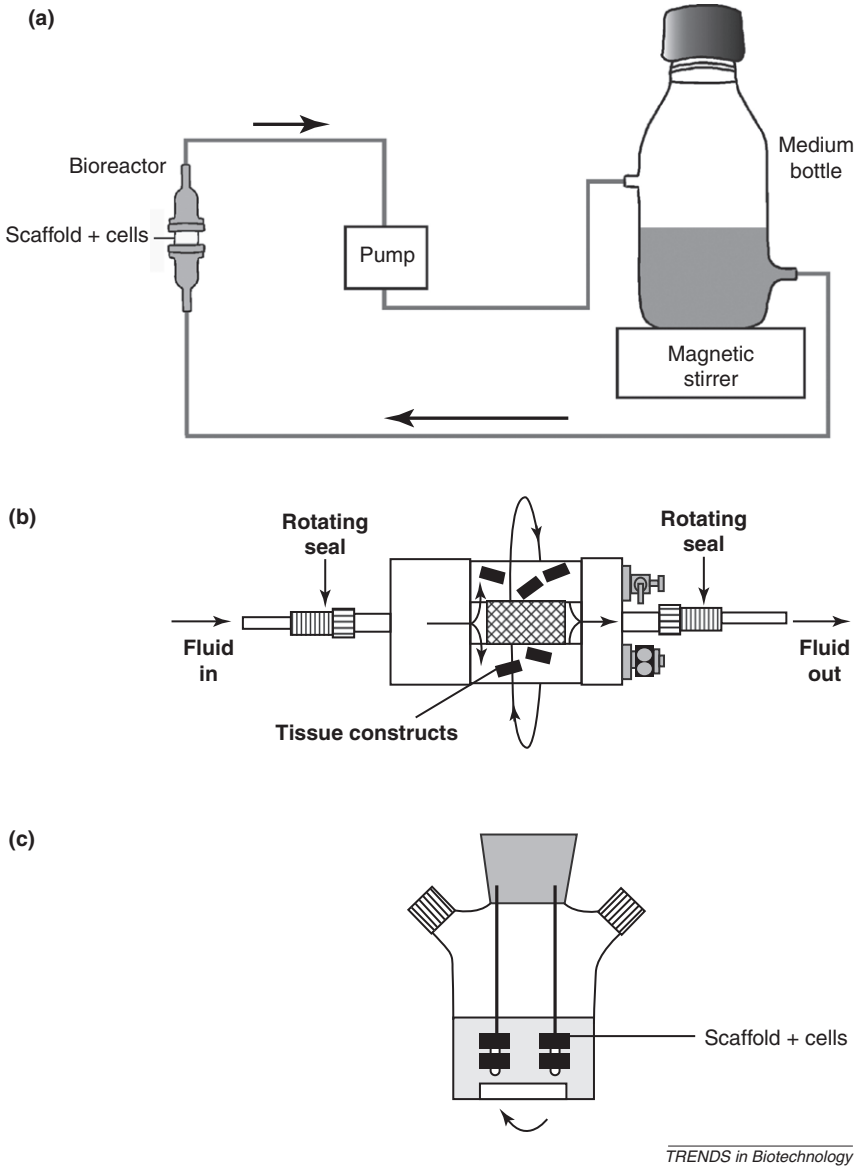

Figure I. Examples of bioreactor configurations suitable for cartilage tissue engineering using porous cell-seeded scaffolds. **(a)** Direct perfusion bioreactor (from [68]. Copyright John Wiley & Sons). **(b)** Perfused rotating bioreactor (from [69]. Copyright Academic Press). **(c)** Spinner flask system (from [69]. Copyright Academic Press).

influences chondrogenic gene expression [48]. Mechanobioreactors designed to deliver long-term mechanical stimuli such as dynamic tension or compression have also been developed [49,50]. The variety of bioreactors tested reflects to a certain extent our lack of understanding of the precise mechanical, hydrodynamic and mass transfer conditions required for chondrogenesis. At present, there is no clear picture of which bioreactor configuration, if any, is most beneficial for chondrogenic development. It is likely that many reactor types will be able to deliver the culture conditions required once those conditions have been identified.

For *in vitro* cartilage production, access to appropriate growth factors, transcription factors and other soluble signaling molecules is likely to be necessary but not sufficient for synthesis of functional matrix. There is a need now to include physical factors in genomic and other omics studies to establish the links between the physical environment, chondrogenic gene expression and cartilage synthesis. As well as gene expression, the physical environment can modulate the activity of growth factors commonly used to induce chondrogenesis [43,51,52], which reveals crosstalk between the control networks responsive to physical and chemical stimuli. When all the physical environmental factors related to the stem cell niche are added to the plethora of biochemical regulators already known to affect chondrogenesis (Table 2), arguably, the number of chemical, physical and biological variables requiring control for cartilage tissue engineering becomes too great to define or monitor for any practical purpose. In these circumstances, systems biology approaches and mathematical simulations that incorporate signaling effects from the physical environment will be useful in identifying the essential relationships operating between the physical and biochemical milieus.

Differentiation plasticity and heterogeneity

Several fundamental scientific advances have been made as a result of recent research into the molecular biology of stem cells. Not least of these are the discoveries that reprogramming of fully differentiated mesenchymal stem cells is possible [53], probably via dedifferentiation [54], that tissue-derived stem cells can differentiate across embryonic germ layers [55], and that clonal cell populations derived from single mesenchymal stem cells exhibit multilineage potency and express multiple cell phenotypes [56]. Heterogeneity, including the coexistence of progenitor cells committed to different differentiation pathways, seems to be an intrinsic feature of tissue-derived stem cells independent of donor-related factors and variations in laboratory protocols [56–58]. Heterogeneity and plasticity contribute to the variability of performance of *in vitro* stem cell cultures and introduce an element of unpredictability into studies aimed at determining the molecular basis of chondrogenesis. The 'transcriptional noise' observed in clonal stem cell populations reflects this cell-to-cell phenotypic variability. Even when progeny are derived from a single stem-cell clone, maintenance of cell individuality is a robust tendency so that over a period of a week or more, the population reverts to heterogeneity. This phenomenon has been attributed to slow fluctuations within the transcriptome and the prevalence of metastable states within each cell type that influence cell fates over an extended period of time [59]. While providing considerable flexibility, in that mesenchymal stem cells can therefore be directed into multiple lineages, these features also manifest themselves in the apparently stochastic or noise-driven nature of cell fate decisions in stem cell cultures [60].

Stem cell heterogeneity and plasticity represent significant problems in tissue engineering. For cartilage production, as well as demonstration of chondroinduction in stem cells, it is also necessary to show at the same time that nonspecific cell programming along other lineages is substantially downregulated or turned off. This is essential to avoid the formation of undesirable or rogue tissues in constructs implanted *in vivo*. Chondrogenic cultures of mesenchymal stem cells have been shown to express the bone markers osteopontin and ALP [61], adipocyte and chondrocyte markers are expressed during osteogenic induction [53], and genes characteristic of osteoblasts and chondrocytes are expressed during adipogenesis [53]. Therefore, although aggrecan and collagen type II may be expressed and synthesized by chondroinduced stem cells *in vitro*, other differentiation markers unrelated to cartilage are also likely to be generated. Together with the recognition that cartilage gene expression does not necessarily result in the synthesis of cartilage matrix (Box 2), this indicates that chondrogenic differentiation cannot be characterized meaningfully by measuring only the expression levels of selected cartilage genes.

Problems with stem cell heterogeneity are especially evident when tissue engineering is used to produce composite constructs containing cells of different tissue-specific phenotype. For example, mesenchymal stem cells could be used to produce osteochondral tissues composed of distinct cartilage and bone layers, but this requires the cells to commit to and maintain distinct differentiation pathways within the two layers. Generation of bone within the cartilage layer, or cartilage within the bone layer, is undesirable. Studies have revealed unwanted nonspecific differentiation in these systems, such as mineralization or significant ALP activity in the chondroinduced regions and GAG or collagen type II production in the osteoinduced regions [62,63]. Methods for isolating more functionally homogeneous mesenchymal stem cell populations and/or the development of differentiation triggers with greater lineage specificity are needed to reduce the risk of unpredictable differentiation outcomes. This should also minimize the phenotypic instability and variability of clinical results observed after *in vivo* application of stem cells [64].

Other cell sources for cartilage production

It is possible that neither differentiated chondrocytes nor mesenchymal stem cells are the optimal starting cell type for cartilage tissue engineering. *In vivo*, these cells mediate normal wound responses and are responsible for the formation of scar tissue. Persistent expression of collagen type I and the production of fibrocartilage by cultured chondrocytes and chondroinduced stem cells could be a reflection of the role these cells play in healing damaged

cartilage. Such wound responses are undesirable in tissue engineering because the repair tissues generated are not fully functional.

To avoid these problems and any other consequences of partial cellular programming, application of embryonic or induced pluripotent stem cells that are at a less advanced stage of differentiation may be beneficial. However, as well as the ethical, immunorejection and tumorigenicity problems that would affect any practical implementation of these cells, a disadvantage of this strategy is that additional differentiation and developmental events must be directed and controlled *in vitro* to achieve lineage selection and tissue synthesis. Nevertheless, stepwise directed differentiation of human embryonic stem cells along known developmental pathways has been successful in generating several tissue-specific cell types, including immature chondrocytes [65]. The utility of induced pluripotent stem cells for cartilage production is yet to be demonstrated [66]. It has been shown that these cells retain some degree of epigenetic memory of their somatic origins, and thus give source-dependent differentiation outcomes [67], so the same difficulties with misdirected differentiation pathways already found using mesenchymal stem cells may also affect application of induced pluripotent stem cells in tissue engineering.

Concluding remarks and future perspectives

Omics studies are enabling us to develop a deeper appreciation of the biochemical and physiological complexity of differentiation and tissue development. However, culture systems for producing functional tissues outside of the body have not yet been improved substantially by the current emphasis on investigating the molecular basis of stem cell differentiation. In many ways, this recent research has highlighted the extent of the difficulties affecting *in vitro* cartilage development.

The role of the physical environment in determining cell and tissue phenotype has been largely ignored in stem cell studies, even though mounting evidence indicates that physical processes are crucial in directing differentiation. New technology for tissue engineering based on advanced understanding of the responses of stem cells to physical stimuli may deliver phenotype stability, homogeneity and maturation, all of which are lacking using current culture techniques. To elucidate the links between physical variables and the regulatory networks functioning inside cells, a multidisciplinary approach is required to bring molecular scientists together with biomaterials, bioreactor and cell-culture scientists and engineers.

Even if this were achieved, however, the number of physical and chemical variables now known to affect differentiation and tissue development is overwhelming. The nonlinearity of biosignaling networks and the operation of seemingly stochastic elements in cell fate determination point to additional complexity that has not yet been fully revealed. A completely rational and deterministic approach to *in vitro* tissue synthesis may therefore prove difficult. This does not mean, however, that new technology for tissue engineering cannot be developed: things can be made to work without us fully understanding them. Within the richness of biochemical detail emerging about differentiation, the challenge in the next few years will be to extract the key elements and distinguish what is essential from what is only associated with tissue development or responsible for its robustness. Exploitation of this new knowledge has barely begun. Even so, it is already clear that the relatively simplistic approaches of the past based on application of biochemical induction factors and only a vague understanding of the influence of the physical environment can no longer form the basis of standard protocols for tissue engineering.

At present, the only known route for successful generation of most functional tissues and organs is embryogenesis. The potential of tissue engineering methods that recapitulate native processes of differentiation and morphogenesis has been recognized [9–11]. However, development is itself an extremely complex phenomenon and endeavoring to reproduce it fully *in vitro* is unrealistic. It is likely that one-step culture processes, in which stem cells are expected to commit to a specific lineage, differentiate fully and produce functional tissues in response to a single set of environmental conditions, will be replaced by more complex, multistage production systems that implement a progressive, stepwise approach to tissue formation, mimicking embryogenic development. Knowledge of the appropriate biochemical and morphological markers for successful completion of each developmental stage is required using this approach. Multistep experimental protocols that recapitulate some aspects of development have been used to produce immature chondrocytes from human embryonic stem cells without the expression of hypertrophy markers; however, this is a long way from synthesis of functional cartilage, especially as heterogeneity of gene expression remained a characteristic of the cell population generated [65]. As synthesis of tissue matrix takes place in the embryo within an increasingly complex array of cell and tissue interactions that could prove impossible to reproduce faithfully *in vitro*, mimicking of developmental pathways may be a feasible approach only for recapitulating early-stage differentiation events.

The idea of quick commercial success in tissue engineering seems overly optimistic now given the weight of complexity being revealed about the regulation of cell differentiation and organogenesis. Nevertheless, the goals of tissue engineering and the potential benefits for human health are too worthwhile to give up now. We expect that recent advances in molecular and cell biology will be translated into new tissue engineering technologies within the next few years only if the full range of stimuli that influence cell and tissue phenotypes is taken into account, and after the key elements of intricate and robust developmental processes are identified from the current plethora of information available. The physical environment in cell culture systems must be given the attention it deserves as a crucial regulator of cell function and tissue formation. As we see it, the main barrier to this happening is that a multidisciplinary effort is required.

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