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Stem cell treatments for neurodegenerative diseases: challenges from a science, business and healthcare perspective

“Achim Rosemann*”

Profits in medicine are typically justified by the notion of a ‘social contract’ between science and society: pharmaceutical and biotech companies are permitted to charge high fees, because revenues are reinvested into research and these investments result in new drugs and the improved health of citizens. A central premise of this model is, however, that the development of novel medicines is financially sustainable: new drugs must be sold in high quantities and be subsidized by welfare state arrangements, so that development costs can be transformed into surplus and future discovery. In the emerging field of stem cell medicine, though, it is still uncertain whether these aims can be achieved. In this article I will discuss this situation with a particular emphasis on neurodegenerative disease research. Although the development of marketable stem cell treatments for neurodegenerative and other neurological diseases is now within reach, it is an open question to what extent these emerging therapeutic strategies can be translated into affordable and widely accessible routine applications. The successful marketing of these treatments and, in particular, the facilitation of widespread and equitable access by patients are affected by various challenges. In this article, three central factors that hinder the translation of the therapeutic potential of stem cells into routine, reimbursable healthcare practice will be discussed: unresolved scientific challenges, the risk of unsustainable development costs and the problem of integrating expensive stem cell treatments into national healthcare portfolios.

**Scientific challenges**

Cell therapy applications have now reached advanced clinical trial phases for stroke, Parkinson’s diseases, amyotrophic lateral sclerosis and other neurodegenerative disorders, with potential marketing approvals in the foreseeable future [1]. Advancements in research with induced pluripotent stem cells (iPS), moreover, hold promise for the development of additional treatment options [2]. However, despite a growing body of evidence that stem cell-based strategies for patients with neurodegenerative diseases can be effective, there are many obstacles and, in particular, the facilitation of widespread and equitable access by patients are affected by various challenges. In this article, three central factors that hinder the translation of the therapeutic potential of stem cells into routine, reimbursable healthcare practice will be discussed: unresolved scientific challenges, the risk of unsustainable development costs and the problem of integrating expensive stem cell treatments into national healthcare portfolios.

**KEYWORDS**

- access to medicines
- affordability
- healthcare decisions
- IPS cells
- neurodegenerative diseases
- stem cell medicine

“**For clinical investigators and small-to-mid-size companies that invest in the development of treatments for neurodegenerative diseases, the high costs of drug development are difficult to cover.”**
to be overcome before these treatments can be approved as safe and successful routine applications. These challenges range from the reliable characterization of cell types, to the development of scalable methods for accurate cell production, to difficulties regarding cell purification, directed differentiation and the control of cell development following transplantation [3]. All of these issues are linked to potential health risks for human research participants and patients, such as tumorigenicity and functional interference due to undesired cell migration and cell activity [4]. Another problem concerns immunological incompatibility between transplanted cell derivatives and potential recipients, which is particularly salient in the case of human embryonic stem cell treatments [5]. In response to this problem, many researchers focus now on autografts of cells derived from iPS cells, which allow to mitigate the problem of allogeneic immune rejection to some extent. The need to address these issues and to produce stem cell products in a scalable and GMP-compliant way that is safe for patients requires large amounts of finance that further increases development and future application costs.

The risk of unsustainable development costs
For clinical investigators and small-to-mid-size companies that invest in the development of treatments for neurodegenerative diseases, the high costs of drug development are difficult to cover. Geron Corporation, for example, which developed the world’s first human embryonic stem cell (hESC) product that entered clinical trials, had to invest about US$200,000,000 in its hESC program, before US FDA approval for a Phase I trial could be obtained. Unfortunately, the trial had to be halted after only five patients, for financial and strategic reasons [6]. It is important to note, though, that – because Geron’s groundbreaking work took place in an early phase of hESC research – the costs were much higher than for the development of subsequent hESC products [7]. The reason: drug development costs decrease as a new technology advances, and as regulatory frameworks are getting more mature. Nevertheless, in high-income countries the obligatory conduct of Phase I to Phase III trials, and subsequent product release costs do often run up to hundreds of millions of US dollars [8]. The high expenses of developing stem cell therapies are reflected in the costs of treatment delivery. It is expected that the fees for stem cell-based treatments for neurodegenerative diseases will be between US$30,000 and 100,000 [9].

For individualized and technologically more complex iPS cell treatments estimates lie around US$200,000 or more [10]. These high costs will clearly decrease the range of potential users. More sophisticated cell products that involve iPS cells or human embryonic stem cells, are especially likely to remain unavailable to large numbers of patients. In the light of this situation, the risk of financial unsustainability is high. It remains to be seen whether development costs can be amortized, and sustainable profits can be generated.

Unaffordability to national healthcare systems
Healthcare systems worldwide are under pressure. Highly finite financial resources and demographic transformations, in particular the shift toward ageing societies, have resulted in cuts in healthcare budgets and cost-containment measures [11]. These changes, together with the availability of new but costly medicines, devices and diagnostics tools, require tough decisions about which medical products and services can be integrated into national healthcare portfolios. These decisions are based on scientific, social and economic value judgments that aim to maximize equitable access to the most efficient and cost-effective treatments. This means that, often, life-saving but too expensive medicines are not covered by national healthcare systems, especially if the money can yield greater benefits in other ways [12]. It is an open question to what extent stem cell treatments will pass the cost-effectiveness thresholds that are set by healthcare administrators in different countries. Due to the high application costs, stem cell therapies may be beyond the financial capacity of national healthcare systems. This applies to both economically developed and less developed countries, but the barriers in terms of access and affordability will be more profound in low-to-middle-income countries [9]. As recently pointed out by the stem cell biologists Viviane Tabar and Lorenz Studer, given the high costs that are associated with iPS and other pluripotent cell technology, the integration of these treatments into routine, reimbursable medical practice is highly unlikely [3]. In Japan, though, various measures have been taken in the last 2 years to reduce the production costs of iPS cell therapies. A fast track approval system for the commercialization of stem cell treatments was approved in 2013 [13], and the inventor of iPS
cells, Shinya Yamanaka, has initiated the creation of a cache of iPS cells that allows for more rapid and less costly forms of clinical application than derivation of IPS cells from individual patients [14]. Whether these measures will succeed in reducing the clinical costs of iPS and other technologically complex cell technologies, and allow for the passing of the cost–effectiveness thresholds of public healthcare services remains to be seen.

**Conclusion**
If the implementation costs for stem cell-based treatments for neurodegenerative diseases cannot be reduced, and the cost–effectiveness criteria of public healthcare services cannot be met, this emerging group of therapies may be affordable only to a smaller group of wealthy patients. This development runs counter to the principles that have guided the relationship between medicine and society in most high-income countries during the last decades. It provokes fundamental questions about distributive justice, and the level of inequality that will be acceptable to national healthcare services. Debates on the reimbursement of stem cell treatments are reminiscent to discussions on the costs of drugs for orphan diseases. Similar to many orphan drugs, several stem cell-based therapies will probably not pass the cost–benefit evaluation of national healthcare systems, and the advantages for overall population health are therefore likely to be limited [15]. For some disease indications, however, the high expected expenses of stem cell treatments are likely to be outweighed by the enormous societal cost of these diseases. This may apply in particular to therapies for care-intensive and chronic neurodegenerative disorders with a high prevalence, such as Alzheimer’s, stroke and Parkinson’s diseases. At this moment, though, approved treatments for these conditions still do not exist. Healthcare systems and funding agencies worldwide will have to confront these questions, and reappraise whether investments in the development of pluripotent stem cell treatments offer really the best option and value for money that public and private health services can offer to patients.

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10 Knoepfler P. Yamanaka on making iPS cells from each patient: ‘in reality, we cannot do that’. www.ipscell.com/2012/11/yamanaka-on
An increased understanding in the pathophysiology of chronic inflammatory diseases, such as rheumatoid arthritis, reveals that the diseased tissue and the increased presence of macrophages and other overexpressed molecules within the tissue can be exploited to enhance the delivery of nanomedicine. Nanomedicine can passively accumulate into chronic inflammatory tissues via the enhanced permeability and retention phenomenon, or be surface conjugated with a ligand to actively bind to receptors overexpressed by cells within chronic inflammatory tissues, leading to increased efficacy and reduced systemic side-effects. This review highlights the research conducted over the past decade on using nanomedicine for potential treatment of rheumatoid arthritis and summarizes some of the major findings and promising opportunities on using nanomedicine to treat this prevalent and chronic disease.

Keywords: chronic inflammation • enhanced permeability and retention • liposomes • nanomedicine • nanoparticles • rheumatoid arthritis

Introduction to chronic inflammatory disease
Inflammation is a normal response that protects tissues from infection or injury. The normal cycle of acute inflammation includes the activation of inflammatory mediators and recruitment of monocytes from circulation to remove foreign pathogens at the inflammation site. The resolution of inflammation consists of downregulation of proinflammatory mediators, release of anti-inflammatory mediators and the removal or clearance of apoptotic cells by phagocytes (i.e., efferocytosis) [1–4]. Acute inflammation promotes tissue repair (through the production of anti-inflammatory mediators), removes damaging pathogens and restores normal tissue functions; however, when the inflammatory trigger is not cleared or is persistent, or when the inflammation is nonresolving for other reasons, chronic inflammation can arise. Chronic inflammation involves the ongoing induction of proinflammatory mediators, infiltration of monocytes into the tissue and ultimately leads to tissue damage. This non-resolving inflammatory response, which may include overexpressed anti-inflammatory mediators, damaged tissues, necrotic monocytes that are not cleared through the lymphatic system and other factors, can become an inflammatory trigger in itself, and can result in an adaptive immune response [5–7]. Genetic factors, environmental triggers as well as adaptive immune response can lead to chronic inflammatory disease [8–10].

Rheumatoid arthritis (RA), inflammatory bowel disease (IBD), chronic obstructive pulmonary disease (COPD) and systemic lupus erythematosus (SLE) are examples of chronic inflammatory diseases. In this review, RA and its therapies will be highlighted. RA affects approximately 1.0% of the population in developed countries, affecting women three-times more than men [11]. The costs associated with disease management in the USA have been reported as US$19.3–39.2 billion per year for RA, with approximately 30% of that costs covered by patients [12–17].
The range of costs can be associated with direct and indirect medical expenses, costs of therapy chosen and the duration of disease. The CDC estimates 15,600 RA hospitalizations annually, further illustrating the prevalence and extent of care required for managing this disease [18].

The clinical presentation of RA is characterized by synovial inflammation, which can lead to deformation, bone erosion as well as loss of joint function. Other clinical manifestations often include the presence of rheumatoid factor (RF) and anticitrullinated protein antibody (ACPA) in the blood, muscle soreness and joint tenderness, as well as increased risk for cardiovascular, pulmonary and skeletal disorders [8]. The etiology of the disease is not completely understood; however, connections have been made to the HLA genes as well as other risk factors [4, 19].

Most chronic inflammatory diseases can be controlled, but not cured, with currently available therapies. The current standard of care (SOC) for RA consists of: anti-inflammatory drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids; disease-modifying antirheumatic drugs (DMARDs); biologic agents and surgery as needed. NSAIDs are primarily used to reduce minor inflammation and relieve pain; however, they do not reduce joint damage and are associated with significant side-effects including gastrointestinal bleeding, fluid retention and increased risk of heart disease. Corticosteroids have beneficial anti-inflammatory and immunosuppressive properties, making them applicable for bridging therapy with other agents, as low-dose therapy for continuous treatment, as high-dose therapy for the treatment of flares or administered intra-articularly for symptomatic relief. Their use, however, is greatly limited by adverse effects such as adrenal suppression, glucose intolerance and increased susceptibility to infections. DMARDs include methotrexate (MTX), hydroxychloroquine, sulfasalazine and leflunomide. Early introduction of these agents has been shown to provide a more favorable outcome in patients, and they are known to reduce disease progression. However, these agents are not without side-effects, namely gastrointestinal complications, liver toxicity and hematologic adverse events. Biologic agents directly target components of the immune response, including pro-inflammatory cytokines and immune cells. Examples of these proinflammatory cytokines include TNF-α, which is the therapeutic target of infliximab, etanercept, adalimumab, golimumab, certolizumab; IL-1, the therapeutic target of anakinra; and IL-6, the therapeutic target of tocilizumab. Biologics also target B cells (rituximab) and block the costimulatory signal required for T-cell activation (as seen with abatacept). Despite their efficacy, biologics are also associated with significant adverse effects, specifically increased risk of infections (e.g., tuberculosis) and certain types of cancers [20, 21].

To impact the progression of disease, the pathogenesis of disease must be understood. Although the etiology of disease remains unknown, great strides in research have led to the identification of agents involved in the initiation and propagation of the inflammatory response. Proinflammatory cytokines TNF-α, IL-1 and IL-6 have been the targets of biologic therapies such as Humira (adalimumab), a TNF-α inhibitor used for the treatment of RA, Crohn’s disease and ulcerative colitis [20–22]. Intracellular signal pathways have also been studied, with a focus on protein kinases. Kinase inhibitors block protein phosphorylation, thereby preventing the activation of transcription factors that control the release of proinflammatory mediators, including the aforementioned TNF-α, IL-1 and IL-6. Protein kinases including JAK, Syk, P13K and p-38 MAPK have been identified in the signal transduction pathway for RA [20, 21, 23–25]. Xeljanz (tofacitinib), the JAK inhibitor, has been approved for use in patients with RA.

Overview of nanomedicine

Nanomedicine is defined as the application of nanotechnology in the diagnosis, treatment or prevention of disease. Nanomedicines may include drug-loaded liposomes, nanoparticles, polymeric micelles, nanogels and nanocapsules [26]. In addition, polymer–drug conjugates, polymer–protein conjugates and antibodies are all classified as nanomedicines [26]. Nanomedicines can be designed to: protect the therapeutic agent from degradation, remain in blood circulation longer, be tailored for macrophage uptake or targeted to certain receptors and permeate through certain diseased tissues as interendothelial cell gaps are generally 1–2 nm in healthy tissues [27–30], but can be up to 600 nm in diseased tissues, such as inflamed joints [31, 32].

Nanomedicines can be prepared with polymers, lipids as well as inorganic nanostructures. Lipid-, polymer-, and hybrid lipid–polymer-based nanomedicines are often utilized for intravenous (iv.) administration and include, but are not limited to, liposomes, PEGylated liposomes, polymericosomes, micelles, dendrimers and hydrogel nanoparticles. Nanoparticle system should be designed considering: the properties of the active molecule to be delivered, the biological target, the environment prior to reaching the target and the environment at the target sites [33, 34]. Hydrophobic and hydrophilic properties of polymers and lipids can be manipulated to encapsulate hydrophobic or hydrophilic active moieties. The types of polymers
and lipids used can be determined based on compatibility with the active drugs or modify drug-release property. Ligands or antibodies can be conjugated onto the nanoparticles to facilitate active targeting, if a known receptor is overexpressed in diseased tissues, which will be discussed in more details later. Furthermore, PEG chains can be covalently conjugated onto nanoparticles, also known as PEGylation, to decrease clearance of the nanoparticles when in circulation by providing a hydrophilic and steric barrier against opsonization [34,35].

**Inflammatory tissue as a target for drug delivery**

There has been a surge in the use of nanomedicine for drug delivery in the treatment of cancer, as solid tumor environment consists of ‘leaky’ vasculature from gaps in the endothelial cell lining and fenestrations that allow for higher permeability of relatively large molecules and particles. Additionally, the presence of other mediators in the diseased tissue can also increase permeability. For example, TNF-α elicits monocyte recruitment from circulation and stimulates additional pores in the endothelial lining. This enhanced permeability paired with an impaired lymphatic drainage system in tumor tissues is known as the enhanced permeability and retention (EPR) effect [34,36]. Utilizing the EPR phenomenon for targeted drug delivery may increase the efficacy and reduce the systemic toxicities of potent anticancer agents [37,38].

The process of monocyte recruitment from circulation and the development of endothelial gaps that facilitate plasma leakage into the injured site are characteristic of inflamed tissues. In chronic inflammatory conditions, inflammatory mediators can be overexpressed and persistent, leading to ‘leaky’ vasculature similar to that seen in solid tumors [39,40]. In addition to increased permeability, inflamed tissues also have more activated macrophages or other monocytes that can be utilized as targets for site-specific drug delivery. Moreover, it has been shown that certain cell adhesion molecules (CAMs) are overexpressed on endothelial cells in IBD [41] and that vasoactive intestinal peptide (VIP) receptors are overexpressed in activated synoviocytes in patients with RA [42]. Ligands specific to those overexpressed molecules can be conjugated to nanomedicine to actively target drug to inflammatory tissues.

**Current landscape of nanomedicine for RA**

Adalimumab (Humira), etanercept (Enbrel) and infliximab (Remicade) are currently among the top ten best-selling drugs in the USA. They are biologics used for the treatment of RA and other inflammatory diseases. However, significant adverse effects may occur with the use of these biologics, leaving patients vulnerable to serious infections such as tuberculosis [43]. The use of nanocarriers allows for increased site-specific drug delivery to inflamed tissues, by utilizing the disease state including, but not limited to, enhanced permeability or changes in pH of inflamed tissues and by utilizing monocytes as active targets for drug delivery. For RA, iv., intra-articular (ia.) and subcutaneous (sc.) administration routes will be reviewed as these allow for systemic delivery to circulation with access to ‘leaky’ vasculature, or local administration directly to diseased tissues; thereby, allowing for maximum drug action. Note that this may differ depending on the disease state; for example, when treating IBD, nanoparticles may be dosed orally because the intestine is the diseased tissue for this condition.

Studies of nanomedicines for potential treatment of RA are summarized in Table 1. These include in vitro, in vivo and clinical studies utilizing nanomedicines for targeted drug delivery to diseased tissues in RA animal models or patients. This summary includes information from searches of multiple databases of scientific literature, including PubMed and ScienceDirect as well as for clinical trials [44]. These searches were limited to publications and clinical applications within the last 10 years. These studies evaluated the use of passive or active targeting for drug delivery, as well as the ability to increase the efficacy of existing therapies by utilizing nanomedicines.

**Taking advantage of enhanced permeability**

The passive targeting of nanomedicines to inflamed tissues based on enhanced permeability has been supported by various in vivo biodistribution studies [51,56.59,63,70]. Ishihara et al. showed that PEGylated polymersomes encapsulated with the glucocorticoid betamethasone preferentially accumulated in inflamed joints in a mouse model of antibody-induced arthritis. The high accumulation correlated with reduction in arthritic score, as well as reduced expression of proinflammatory cytokine, IL-6. In vivo imaging showed that the accumulation of the polymersomes in the joints maintained for up to 96 h, which led to a sustained therapeutic effect for 8 days [56].

Glucocorticoids are often utilized for patients with RA, and are considered potent anti-inflammatory agents; however, the exact mechanism of action of this class of drugs is not completely understood. Encapsulation of them into liposomes or polymersomes allows for more local delivery and accumulation to inflammation sites due to the EPR effect, thereby reducing systemic side-effects and enhancing therapeutic efficiency. Hofkens et al. showed that prednisolone
Table 1. Nanomedicines for the treatment of rheumatoid arthritis: *in vitro*, *in vivo* and clinical trials.

<table>
<thead>
<tr>
<th>Type of therapy</th>
<th>Drug</th>
<th>Nano-DDS</th>
<th>Route</th>
<th>Targeting mechanism</th>
<th>Phase</th>
<th>Model†</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSAID</td>
<td>Piroxicam</td>
<td>Liposomes</td>
<td>NA</td>
<td>Macrophage uptake</td>
<td>Predinical, <em>in vitro</em></td>
<td>RAW264.7 macrophages</td>
<td>[45]</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nimesulide</td>
<td>Polymeric nanoparticles</td>
<td>ia.</td>
<td>Passive</td>
<td>Predinical</td>
<td>NA</td>
<td>[46]</td>
</tr>
<tr>
<td>Gold salts</td>
<td>Gold salts</td>
<td>Nanoparticles</td>
<td>NA</td>
<td>Macrophage uptake</td>
<td>Predinical, <em>in vitro</em></td>
<td>RAW264.7 macrophages</td>
<td>[47]</td>
</tr>
<tr>
<td>Corticosteroid</td>
<td>Betamethasone</td>
<td>PEGylated polymersomes</td>
<td>iv.</td>
<td>Passive</td>
<td>Predinical, <em>in vivo</em></td>
<td>AA rats; AbIA mice</td>
<td>[56]</td>
</tr>
<tr>
<td>Corticosteroid</td>
<td>Dexamethasone</td>
<td>Liposomes</td>
<td>iv.</td>
<td>Passive</td>
<td>Predinical, <em>in vivo</em></td>
<td>AA rats; CIA mice</td>
<td>[57,58]</td>
</tr>
<tr>
<td>Corticosteroid</td>
<td>Dexamethasone</td>
<td>PEGylated liposomes</td>
<td>sc.</td>
<td>Active (<em>αvβ3</em> receptor)</td>
<td>Predinical, <em>in vivo</em></td>
<td>AA rats</td>
<td>[59]</td>
</tr>
<tr>
<td>Corticosteroid</td>
<td>Dexamethasone</td>
<td>PEGylated liposomes; liposomes</td>
<td>NA</td>
<td>Passive</td>
<td>Predinical, <em>in vitro</em></td>
<td>Leukocytes, fibroblasts, hepatocytes, macrophages</td>
<td>[61]</td>
</tr>
<tr>
<td>Folic acid antagonist (immunosuppressant)</td>
<td>Methotrexate</td>
<td>Dendrimers</td>
<td>iv.</td>
<td>Active (folate receptor)</td>
<td>Predinical, <em>in vivo</em></td>
<td>CIA rat</td>
<td>[62]</td>
</tr>
<tr>
<td>Folic acid antagonist (immunosuppressant)</td>
<td>Methotrexate</td>
<td>Lipid nanoemulsions</td>
<td>ia.</td>
<td>Passive</td>
<td>Predinical, <em>in vivo</em></td>
<td>AIA rabbits</td>
<td>[63]</td>
</tr>
<tr>
<td>Folic acid antagonist (immunosuppressant)</td>
<td>Methotrexate</td>
<td>Polymeric nanoparticles</td>
<td>NA</td>
<td>Active (anti-CD64 antibody)</td>
<td>Predinical, <em>in vitro</em></td>
<td>RAW264.7 macrophages</td>
<td>[64]</td>
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<td>Folic acid antagonist (immunosuppressant)</td>
<td>Methotrexate</td>
<td>Polymeric nanocomplexes</td>
<td>NA</td>
<td>Cellular uptake</td>
<td>Predinical, <em>in vitro</em></td>
<td>HepG2</td>
<td>[65]</td>
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<td>Folic acid antagonist (immunosuppressant)</td>
<td>Methotrexate</td>
<td>Polymeric nanoparticles; silica nanoparticles</td>
<td>ip.</td>
<td>Passive</td>
<td>Predinical, <em>in vivo</em></td>
<td>PIA rats</td>
<td>[66]</td>
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<td>Cyclosporine</td>
<td>Polymeric micelles (polysialic acid)</td>
<td>NA</td>
<td>Passive</td>
<td>Predinical, <em>in vitro</em></td>
<td>SW982 cells</td>
<td>[67]</td>
</tr>
</tbody>
</table>

†Model for *in vitro* or *in vivo* evaluation.
AA: Adjuvant arthritis; AbIA: Antibody-induced arthritis; CIA: Collagen-induced arthritis; DAPT: N,N-(3,5-difluorophenacetyl)-L-alanyl-L-phenylglycine t-butyl ester; DDS: Drug delivery system; ia.: Intra-articular; ip.: Intraperitoneal; iv.: Intravenous; KRN: Serum-induced arthritis; NA: Not applicable; NSAID: Nonsteroidal anti-inflammatory drug; PIA: Pristane-induced arthritis; sc.: Subcutaneous; TRAIL: TNF-related apoptosis inducing ligand; VIP: Vasoactive intestinal peptide.
Table 1. Nanomedicines for the treatment of rheumatoid arthritis: *in vitro*, *in vivo* and clinical trials (cont.).

<table>
<thead>
<tr>
<th>Type of therapy</th>
<th>Drug</th>
<th>Nano-DDS</th>
<th>Route</th>
<th>Targeting mechanism</th>
<th>Phase</th>
<th>Model†</th>
<th>Ref.</th>
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<td>Fragment TNF-α inhibitor</td>
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<td>sc.</td>
<td>Passive</td>
<td>Phase III</td>
<td>NA</td>
<td>[44]</td>
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<td></td>
<td>(immunosuppressant)</td>
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<td>TRAIL</td>
<td>PEGylated nanocomplexes</td>
<td>sc.</td>
<td>Active (ligand, DR 5 receptor)</td>
<td>Predinical, <em>in vivo</em></td>
<td>CIA mice</td>
<td>[68]</td>
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<tr>
<td>agent</td>
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<tr>
<td>Immunomodulatory</td>
<td>TRAIL</td>
<td>Liposomes (conjugated)</td>
<td>ia.</td>
<td>Passive</td>
<td>Predinical, <em>in vivo</em></td>
<td>AIA rabbits</td>
<td>[43]</td>
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<td>agent</td>
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<td>Curcumin</td>
<td>Nanoemulsions</td>
<td>NA</td>
<td>Macrophage uptake</td>
<td>Predinical, <em>in vitro</em></td>
<td>RAW264.7 macrophages</td>
<td>[69]</td>
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<td>agent</td>
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<td>Angiogenesis inhibitor</td>
<td>Fumagillin</td>
<td>Lipid nanoparticles</td>
<td>iv.</td>
<td>Active (α, β3 receptor)</td>
<td>Predinical, <em>in vivo</em></td>
<td>KRN mice</td>
<td>[70]</td>
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<td>DAPT</td>
<td>Polymeric nanoparticles (hyaluronic acid)</td>
<td>iv.</td>
<td>Passive</td>
<td>Predinical, <em>in vivo</em></td>
<td>CIA mice</td>
<td>[71]</td>
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<td>PEGylated lipid micelles</td>
<td>sc.</td>
<td>Passive and active (VIP)</td>
<td>Predinical, <em>in vivo</em></td>
<td>CIA mice</td>
<td>[42]</td>
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<td>Gene therapy</td>
<td>tgAAAC94 (TNF-α silencing)</td>
<td>Adeno-associated virus (AAV)</td>
<td>ia.</td>
<td>Passive</td>
<td>Phase II</td>
<td>NA</td>
<td>[44,72]</td>
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<tr>
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<td>PEGylated liposomes</td>
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<td>CIA mice</td>
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<td>Biologic</td>
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<td>Active (VIP)</td>
<td>Predinical, <em>in vivo</em></td>
<td>CIA mice</td>
<td>[82]</td>
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†Model for *in vitro* or *in vivo* evaluation.

AA: Adjuvant arthritis; AbIA: Antibody-induced arthritis; AIA: Antigen-induced arthritis; CIA: Collagen-induced arthritis; DAPT: N-((3,5-difluorophenacetyl)-(S)-alanyl)-S-phenylglycine t-butyler ester; DDS: Drug delivery system; ia.: Intra-articular; ip.: Intraperitoneal; iv.: Intravenous; KRN: Serum-induced arthritis; NA: Not applicable; NSAID: Nonsteroidal anti-inflammatory drug; PIA: Pristane-induced arthritis; sc.: Subcutaneous; TRAIL: TNF-related apoptosis inducing ligand; VIP: Vasoactive intestinal peptide.
phosphate encapsulated in PEGylated liposomes was able to downregulate the activation of proinflammatory macrophages and upregulate anti-inflammatory macrophages in vitro; however, only the downregulation of proinflammatory macrophages was observed in vivo [51]. The authors also conducted biodistribution studies to confirm that after iv. or sc. administration, the liposomes extravasate through leaky vasculature into synovial tissues and are engulfed by macrophages within the inflamed tissues [50], further supporting the utilization of the enhanced permeability for targeted delivery of anti-inflammatory agents. After macrophage uptake, significant reductions were seen in the expression of proinflammatory cytokines including TNF-α, IL-1β, IL-8 as well as CD86 protein, giving insight into the mechanism of action of the prednisolone phosphate [51]. Because of the promising in vivo results, safety studies were conducted for repeat dosing of the liposomal prednisolone phosphate, as well as dose range finding. It was concluded that the safety profile of the glucocorticoid benefited from the liposomal formulation, and that the effective dose and dose frequency of the glucocorticoid could be reduced in animal models by as much as tenfold; showing comparable efficacy with four daily injections of 10 mg/kg of free drug to a single dose of 1 mg/kg prednisolone phosphate in the liposomes [50]. The ability of nanoparticle formulations of glucocorticoids to suppress proinflammatory cytokines such as TNF-α at a lower effective dose and dose frequency may be advantageous to decrease the broader immunosuppression seen with many biologic TNF-α inhibitors on the market. A Phase II clinical study with liposomal prednisone has been conducted, confirming the safety and increased efficacy of the liposomal prednisone relative to free drug.

Ulmansky et al. used an adjuvant arthritis (AA) rat model to evaluate two formulations of PEGylated liposomes, one containing methylprednisolone and the other betamethasone, against free drug as well as biologic TNF-α inhibitors, etanercept and infliximab. Their in vivo study results showed that the liposomal formulations led to a significant decrease in proinflammatory mediators and a longer duration of therapeutic effect when compared with free drugs or to biological therapies; indicating a promising path forward toward a more effective and less frequent therapy using glucocorticoid–nanomedicines as an alternative to the immunosuppressant biologic therapies [53].

Selective biodistribution in inflamed tissues due to enhanced permeability and the resultant lower effective dose and longer duration of drug action leading to decreased dose frequency are a recurring theme with nanomedicines in RA models [45, 50, 53, 55, 57, 58]. For example, Ulmansky et al. reported a longer duration of drug action in vivo with a liposomal glucocorticoid formulation, resulting in weekly administration, as opposed to daily injections of the free drug as well a decrease in effective dose ranging from two- to 25-fold [53, 54]. Hwang et al. showed that delivery of α-methylprednisolone using a conjugated cyclodextrin polymer-based nanoparticle formulation reduced the dosing frequency from daily to weekly and the effective dose by up to 100-fold, with a comparable or superior suppression of RA symptoms [55].

**Active targeting for increased site-specific delivery**

Although the accumulation of the nanocarriers in inflamed tissues due to enhanced permeability can help to decrease effective dose and dose frequency, increased knowledge of the disease tissues could lead to even more discriminating biodistribution. Chronic inflammation is characterized by a persistent inflammatory response that includes the infiltration of macrophages, lymphocytes and plasma from circulation eventually leading to tissue damage. In the case of RA, tissue damage is mainly seen in the joints, leading to bone erosion and deformation. The affected joint tissue is characterized by an increased expression of adhesion molecules and chemokines, which can be exploited for active drug targeting. Recent studies indicate that folate receptor-β (FR-β) expression is elevated on activated macrophages in inflamed joints in RA [83]. With this information, Thomas et al. prepared MTX dendrimer nanoparticles with folic acid (FA) as a targeting ligand. MTX is one of the most commonly prescribed DMARDs, alone or in combination with biologic therapy; however, many patients are intolerant to the medication due to significant side-effects. Targeted delivery of MTX may help reduce these side-effects. Thomas et al. showed that the FA-conjugated dendrimer nanoparticles selectively bound to the activated macrophages in vivo and led to significant disease suppression in vivo [62]. Additionally, it was shown that the maximum-tolerated dose (MTD) of the MTX in nanoparticles was 7.5-fold higher than that of the free MTX [62]. The FA as a targeting ligand was also used in chitosan-DNA nanoparticles to enhance the delivery of a plasmid that encodes IL-1Ra, a receptor antagonist and natural blocker of IL-1, and the FA-conjugated chitosan-DNA nanoparticles were shown to have less cytotoxic effects than the FA-free nanoparticles, likely due to the active uptake of the particles by activated macrophages, reducing toxicity to other cells [80].

Vascular endothelial cells (VECs) are involved in the recruitment of monocytes from circulation into
inflamed tissues during inflammation. They are also involved in angiogenesis during tissue repair. VECs have certain cell adhesion molecules (CAMs) or growth factor receptors that may be overexpressed in inflamed tissues and could be used as targets for active drug delivery [74,84–86]. Koning et al. evaluated the delivery of dexamethasone with PEGylated liposomes surface-conjugated with a specific peptide ligand to target the αvβ3 receptor, an integrin overexpressed on VECs in inflammation sites. In an in vivo AA rat model, the targeted liposomes showed a threefold increase in accumulation in the inflammation site, compared with the nonconjugated liposomes, which correlated with a significant decrease in arthritic severity score and a longer therapeutic effect [59]. This increased localization was interesting, as the conjugated liposomes were cleared significantly faster from circulation than the nonconjugated liposomes, showing how effective the targeted liposomes were, even with a shorter circulation time [59]. The same receptor was also targeted for the delivery of an angiogenesis inhibitor, fumagillin and the targeted fumagillin showed a higher affinity to inflamed tissues, decreased leukocytes recruited into the tissues, and suppressed inflammation [70]. In addition, the effective dose of the optimized targeted fumagillin nanoparticle formulation was decreased by eightfold [70].

VIP is a hormone active in the resolution of inflammation; therefore, even though resolution of inflammation is not achieved, VIP receptors tend to be overexpressed in activated macrophages and proliferating synoviocytes in RA. The conjugation of VIP to nanoparticles would allow for active targeting of overexpressed receptors; they could also potentially provide therapeutic value by downregulating proinflammatory cytokines and upregulating anti-inflammatory cytokines [87]. Koo et al. utilized VIP as a targeting ligand to deliver camptothecin (CPT), an anticancer drug that induces cell death, to the overproliferating synoviocytes in the previously inflamed tissue during arthritis with CPT in the VIP-conjugated PEGylated micelles [42]. The dose of CPT used was approximately 100-fold lower than used for cancer therapy, and no systemic toxicity was observed in the CIA mouse model, making this a potentially promising new agent for the treatment of RA [42].

Advancing the SOC
The application of nanoparticles in molecular therapy is a growing area of research, as seen in Table 1. A clinical study using IA-injected adeno-associated virus (AAV) to deliver a TNF agonist gene showed the proof of concept for the use of gene therapy in RA; however, the AAV therapy also resulted in significant side-effects, including joint swelling and discomfort at the injection site [72,88]. siRNA that specifically inhibits the expression of certain genes, such as TNF-α, that are critical in RA development and progression is an interesting alternative to biologic agents such as anti-TNF-α antibodies, because it has the potential to selectively inhibit the expression of proinflammatory genes in targeted cells, while avoiding or minimizing the systemic side-effects associated with current biologics. However, siRNA is largely ineffective when given alone in vivo, and is thus generally formulated into nanocarriers such as liposomes or polymeric nanoparticles to protect the siRNA and deliver it to inflamed sites [89–91]. For example, Scheinman et al. encapsulated STAT1 siRNA into nanoparticles prepared with poly(lactic-co-glycolic) acid (PLGA) and showed a fourfold increase in protection of the siRNA from serum nuclease when compared with naked siRNA after 20 h of incubation in bovine serum [78]. Importantly, the STAT1 siRNA nanoparticles caused partial disease regression in a mouse model of RA, a notable achievement as most therapies act to stop disease progression or to ameliorate symptoms, pointing to the potential of using siRNA in RA therapy [78]. Komano et al. encapsulated TNF-α siRNA using Wrapsomes consisting of a cationic lipid bilayer core that was surrounded by the siRNA complex. The siRNA-cationic lipid bilayer core was then encapsulated by a neutral lipid bilayer and this outer layer was PEGylated to reduce systemic clearance [75,92]. The TNF-α siRNA-Wrapsomes showed therapeutic effect in a mouse model of collagen-induced arthritis when administered at the onset of disease [75]. It was also showed that most of the siRNA in the Wrapsomes was delivered into CD11b+ cells, including macrophages and neutrophils, in inflamed synovium [79]. Khoury et al. complexed TNF-α siRNA with cationic liposomes and showed that the siRNA-cationic lipidome complexes significantly reduced TNF-α secretion, ranging from 50 to 70% inhibition, over the course of 3 weeks and significantly ameliorated the disease in an experimental arthritis model [76].

With increased understanding of disease pathophysiology and the ability to deliver drugs directly to diseased tissues, more diverse agents and ligands
can be evaluated now. For example, the TNF-related apoptosis inducing ligand (TRAIL) is a protein that binds to death receptors (DR) overexpressed on cancer cells, namely DR4 and DR5, inducing apoptosis in these cells without affecting normal, healthy cells [91–95]. This very specific activity makes it a very promising agent for the treatment of cancer, where the SOC is extremely toxic to normal cells, and clinical trials are in progress or completed using TRAIL or its agonistic antibodies in renal cancer, non-Hodgkin’s lymphoma and nonsmall cell lung cancer [44,96–99]. Synoviocytes in RA also overexpress DR5, making it a candidate for TRAIL-induced apoptosis. Kim et al. utilized PEGylated nanocomplex formulations to deliver TRAIL in a CIA mouse model, showing that the PEGylated nanoparticles increased the half-life of the protein by 13-fold and sustained delivery of TRAIL resulted in better efficacy against the disease [68]. Martinez-Lostao et al. used TRAIL conjugated to liposomes for in vivo evaluation in an AA rabbit model. The liposome formulation resulted in significant reductions in synovial hyperplasia, to almost normal values, and reduced angiogenesis and joint inflammation [43].

**Limitations of nanomedicine**

Nanomedicines are not without their limitations, especially for use in chronic conditions. The safety of the nanomaterials must be determined, in addition to the therapeutic agent itself. This may be a costly exercise, reducing the speed or number of nanomedicines that are translated into clinical trials. Additionally, it should also be shown that the nanoparticles or nanocarriers do not themselves incite an inflammatory response. The use of biocompatible, nonimmunogenic and biodegradable materials may be the key to avoiding this type of adverse reactions [90,100].

**Conclusion**

There has been increased interest and applications of nanomedicines in treating RA and other chronic inflammatory diseases. The ongoing development of biocompatible nanomaterials and delivery systems for current antirheumatic agents may ultimately lead to lower effective doses, reduced dose frequency and more effective therapies with less systemic side-effects. Increased understanding of the pathophysiology of RA will not only prompt new ideas to enhance the localization antirheumatic agents in inflamed joints but also open the door to new agents such as siRNA, for which nanocarriers are generally needed to be effective in vivo.

**Future perspective**

In the next few years, more anti-inflammatory agents targeting proinflammatory cytokines will likely be developed to treat RA and other chronic inflammations. Various nanomedicines that lower the dose and dosing frequency of existing anti-inflammatory agents, and thus reducing their side-effects, will likely move to clinical trials and/or clinics. Moreover, increased understanding of the mechanisms underlying RA and other chronic inflammatory diseases will lead to the identification of new drug targets and new drugs.

**Financial & competing interests disclosure**

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No writing assistance was utilized in the production of this manuscript.

**Executive summary**

**Rheumatoid arthritis & its current treatments**

- Chronic inflammations, including rheumatoid arthritis (RA), are diseases that can be controlled, but not cured.
- All current therapies for RA are associated with serious unwanted side-effects, especially after long-term usage.
- Macrophages and cytokines produced by them play a critical role in RA development and progression, and are the targets of many antirheumatic agents.
- Chronic inflammation site has leaky vasculature and impaired lymphatic drainage.

**Nanomedicine in RA therapy**

- Nanomedicine shows promise in the treatment of many diseases, including RA.
- Both small molecular anti-inflammatory agents and large molecules such as siRNA and proteins were delivered using nanocarriers.
- Delivery of anti-inflammatory agents using nanocarriers generally lowers the dose and dosing frequency of the agents, and reduces their side-effects.
- Nanomedicine is not without limitations, and biocompatible materials should be used to prepare nanocarriers to minimize carrier-induced side-effects.
References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

22 Santamaría P. Cytokines and Chemokines in Autoimmune Disease: An Overview. Landes Bioscience, TX, USA (2003).
• Reviews the cell biology and importance of interendothelial gap formation, including the influence of inflammatory mediators and inflammatory disease.


• Reports that nanosized liposomal prednisolone (PLP) at a single dose of 1 mg/kg was comparable in efficacy to four repeated once-daily injections of 10 mg/kg free PLP. Additionally, the low dose of liposomal PLP exhibited suppression of inflammation up to day 21 in antigen-induced arthritis mouse model. This study provides support of ongoing clinical studies using nanosized PEGylated liposomes of prednisolone.


44 ClinicalTrials.gov. www.clinicaltrials.gov


- *In vitro* studies determined time- and concentration-dependent macrophage uptakes of folate-targeted methotrexate (MTX) nanoparticles indicating binding to folate receptor β-expressing macrophage lines; it is known that elevated levels of folate receptor β are expressed on activated macrophages in inflamed joints in rheumatoid arthritis. *In vivo* studies confirmed the significant benefit of the targeted nanoparticles, as well as a reduction in side-effects.


- Reports intra-articular treatment of antigen-induced arthritis rabbit model using lipid nanoemulsions with MTX showing a significant reduction in inflammatory cells in the diseased joint most probably due to slower clearance from the joint when compared with other MTX vehicles and greater uptake than the current folate-targeted MTX commercial formulation.


- Shows the study of new therapeutic agents using nanoparticles. Hyaluronic nanoparticles of γ-secretase (DAPT) significantly reduced tissue damage and neutrophil infiltration in joints, as well as a reduction of proinflammatory cytokines (TNF-α, IFN-γ, MCP-1 and IL-6, -12, -47) providing evidence of therapeutic efficacy for rheumatoid arthritis.


- Polylactic-co-glycolic acid nanoparticles of STAT1 siRNA were used to downregulate IFN-γ signaling; these were further functionalized for more active uptake. Weekly
treatments showed partial disease regression, while other controls continued to show disease progression.


Stem cell-based therapy of corneal epithelial and endothelial diseases

Corneal dysfunction is the second leading cause of blindness. Approximately 10 million patients worldwide are affected by some form of corneal disease. More than 50,000 cornea transplants are performed every year, but this procedure is limited by cornea donation availability. Recently, new cell replacement procedures have been developed to treat a variety of corneal diseases. This review will focus on the recent advances in the use of limbal epithelial stem cells (LESCs) to treat corneal epithelial cell deficiency and improvements in replacing dysfunctional corneal endothelial cells (CECs) with exogenous CECs. Several protocols have been developed to differentiate pluripotent stem cells into LESC- or CEC-like cells, potentially yielding an unlimited source for the cell replacement therapy of corneal diseases.

Keywords: cell transplantation • corneal endothelium • corneal transplantation • limbal epithelial stem cells • stem cell differentiation

The eye is a highly specified organ for light and image perception. From the cornea to the retina, every cell type in the eye serves a specific function including light refraction, accommodation, photoelectrical transduction, electrical signal transmission, etc. Thus, degeneration or malfunction of any cell type can lead to different types of ocular diseases. This minireview will focus on the cornea and the various clinical approaches to treat corneal diseases. Readers who are interested in cell therapy of retinal disorders may refer to a recent excellent review paper by Coffey et al. [1] that describes the recent progress in transplanting retinal pigment epithelial cells to age-related macular degeneration and Stargardt disease [2].

In humans, the outer surface of the eye sits on a protective structure barrier called the cornea, which is composed of five distinct layers (see figure 1). The cornea primarily functions to transmit and refract light while keeping the integrity of anterior chamber.

At the outermost surface, corneal epithelium is composed of several layers of corneal epithelial cells that are connected to each other with tight junctions. Corneal epithelial cells undergo constant self-renewal via basal cell proliferation and differentiation of limbal epithelial stem cells (LESCs) that are found in the corneal limbus located between the transparent cornea and opaque conjunctiva. Any condition that causes the loss or reduction of LESCs to a certain degree will lead to corneal epithelium defect, corneal hazing, or even blindness, which is clinically termed as limbal stem cell deficiency (LSCD). LSCD can be caused by chemical burn or traumatic injury of a large area of cornea surface, hereditary corneal dystrophy and several immune disorders such as Stevens–Johnson syndrome [3].

In contrast to the multiple layers of epithelial cells in the corneal epithelium, the corneal endothelium is comprised of a monolayer of hexagonal endothelial cells. Though a single layer, corneal endothelial cells (CECs) pump extra water to the anterior chamber and regulate the cornea to proper hydration, thus playing a pivotal role in maintaining cornea transparency. CECs also allow small molecules and nutrition to traverse from...
the aqueous humor to the stromal layer, thus contributing to cellular metabolism of avascular stromal cells [4]. CECs barely proliferate in vivo, and the density of CECs decreases with age at the rate of 0.6% per year [5,6]. Upon Fuchs endothelial dystrophy, trauma or a complication of intraocular surgery, etc., which leads to the corneal endothelium’s damage, CECs’ density decreases below 400–700/mm², and the corneal transparency cannot be maintained due to accumulation of fluid anteriorly into the stroma and epithelium layers. Excess fluid not only clouds the cornea but also forms a blister-like structure between the basal epithelium cells, thus affecting vision and causing pain sensations as described for bullous keratopathy.

The promise of stem-cell-based treatments of corneal diseases

Corneal transplantation (or keratoplasty) is usually the first choice of treatment for many corneal diseases in the clinic. These diseases include corneal leukoma, which affect the patients’ visual acuity, bullous keratopathy, advanced keratoconus, etc. Besides penetrating keratoplasty, advanced surgical procedures have also been developed, including limbus transplantation, Descemet’s membrane endothelial keratoplasty (DMEK), and deep laminar keratoplasty. However, hundreds of thousands of patients worldwide are waiting for transplantation surgery due to shortage of corneal donors. Therefore, stem-cell-based treatments have been proposed as a promising way to solve the problem. For example, autologous ex vivo expansion of corneal limbal epithelial cells have been used to treat LSCD. In addition, novel methods of using pluripotent stem cells to differentiate to LESC- and CEC-like cells also hold great promise for treating corneal diseases. It is worth noting that we focus on reviewing corneal epithelial and endothelial cell transplantation in this paper and will not discuss the applications of other types of stem cells such as mesenchymal stem cells and corneal stromal stem cells in corneal stroma regeneration [7,8].

Cell sources for treating LSCD

The self-renewal, migration and differentiation of limbal stem cells is essential for maintaining corneal epithelium structural integrity and repairing corneal damage. One of the most recent advances in the treatment of LSCD is autologous cell transplantation after ex vivo expansion of LESC. LESC are thought to be precursors of corneal epithelial cells [9], and was one of the earliest stem cells applied on clinical applications [10–12]. The concept of LESC was not clear in the early clinical application, and the property of transplanted stem cells were hotly debated [13]. However, it is now well accepted that LESC are present in the limbal biopsy. Furthermore, LESC morphology and putative markers are now routinely used to identify LESC, such as small cell size, high nucleus/cytoplasm ratio and euchromatin rich nuclei [14,15].

Early efforts focused on the identification and validation of LESC markers including OCT4, LGR5, integrins (α9 and β1), NGF receptors (TrkA), CK15, CK19, etc. [16,17]. Ultimately, ABCG2, ΔNp63α, C/EBPδ and Bmi-1 are now accepted as putative LESC markers. ABCG2 is a member of the ATP binding cassette transporters and recognized as a universal marker of stem cells [18–20]. In addition, expression of ABCG2 was also found in a number of cancer cells and appears to also be a marker of cancer stem cells [21,22]. ΔNp63α is a truncated transcriptional variant of the p63 gene, which has six isoforms. High p63 content is present in limbal epithelial cells and suggested as a putative LESC marker [9]. ΔNp63 β and γ isoforms were regarded as promoters to epithelial cell differentiation, and ΔNp63α is accepted as another marker of LESC [23–25]. Bararo et al. [24] showed that coexpression of C/EBPδ, Bmi1 and ΔNp63α can be used to identify resting limbal stem cells. C/EBPδ, but not ΔNp63α, indefinitely promotes holoclone self-renewal and prevents clonal evolution. Recently, it was shown
that ABCB5 is a substantial marker for LESC s \[26\]. ABCB5 was found to coexpress with p63α in human LESC s and play a pivotal role in corneal epithelium development and requirement. All of these proteins are not highly specific markers for LESC s; many different stem cells express some of these markers. Therefore, these markers are often used in combination to identify bona-fide LESC s.

Autologous LESC is an ideal source of corneal epithelial transplantation due to the favorable lack of immune rejection. But, indications for autologous LESC transplantation are limited. The procedure requires some healthy limbal tissue. Therefore diseases such as in Steven–Johnson Syndrome and other diseases, which may cause extensive damage of eye surface bilaterally, cannot be treated by autologous LESC s. Thus, other cell sources are required to treat these difficult cases of LSCD. For example, LESC s from close relatives of patients are a sensible source for transplantation, with the only disadvantage of potential graft rejection. Because LESC s are the obvious target for stem cell differentiation, a wide range of stem cell sources are tested for differentiation into LESC-like cells. Somatic stem cells like bone-marrow–derived mesenchymal stem cells \[27,28\], hair follicle stem cells \[29,30\], dental pulp stem cells \[31\], umbilical cord stem cells \[32\] and skin epithelial stem cells \[33\] have been used to reconstruct the corneal epithelium. Most recently, two studies carefully examined homeostasis of limbal stem cells and found that the Wnt7A-Pax6 axis is required for the development and maintenance of limbal stem cells \[33\]. Furthermore, one group showed that ABCB5 is a major marker labeling limbal stem cells in both human and murine limbal stem cells \[80\].

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) show no obvious advantage in differentiation and transplantation to corneal epithelium compared with somatic stem cells. However, ESCs and iPSCs could be mass-produced due to their unlimited proliferation capacity. Moreover, iPSCs can be considered autologous, which is thought to reduce the immune reaction. Homma et al. first reported the differentiation of ESCs to epithelial progenitor cells and the reconstruction of mice corneal surface \[34\]. Subsequently, ESCs were found to be capable of differentiating into a monolayer of epithelium-like cells \[35,36\]. iPSCs also showed a similar differentiation potential \[37,38\]. Recently, researchers found that proper limbal niche, including specialized extracellular matrix and cytokines, is essential for maintaining LESC and differentiation of corneal epithelial cells \[39\]. Ahmad et al. showed that human ESCs differentiate into corneal epithelial-like cells on collagen IV using medium conditioned by the limbal fibroblasts \[36\]. A variety of cell sources for treat-
ing LSCD are summarized in Table 1, including LESCs, somatic stem cells, and pluripotent stem-cell-derived corneal epithelium lineage cells.

### Cell sources for corneal endothelial diseases

Compared to corneal epithelium, the protocol for ex vivo expansion of autologous CECs is not well established yet. The main reason is due to limited CECs’ proliferative capacity in vitro [40–42], which also results in the smaller number of CECs studies compared with corneal epithelial cells. It is estimated that the adult primary CECs can be passaged around four times, and there is little improvement even with modified medium and supplemented cytokines [43]. Yet, several protocols are developed to promote the proliferation of CECs, including the use of human bone marrow mesenchymal stem-cell-derived conditioned medium [44], or human amniotic epithelial-cell-derived conditional medium [45] (Table 2). In addition, telomerase or Cdk4R24C (constitutively active mutant form of Cdk4) and CyclinD1 transduction into CECs showed in vitro pump function [46,47]. Although no oncogenes were transduced, clinical safety is still a concern for these genetically modified cell lines. Hirata-Tominaga et al. recently studied the important role for LGR5 in maintaining the fate of CECs, and they found that the ligand RSPO-1 could stimulate CECs proliferation in vitro [48]. Gao et al. developed a protocol for fetal CEC culture; however, the authors found that fetal CECs do not exhibit a higher proliferative capacity [49].

Another source for CEC transplantation is corneal precursors (Table 2). Both corneal stromal and endothelial cells contain a significant number of precursors. Yoshida et al. reported isolation of cornea-derived precursors from the mouse corneal stroma which has characteristics of multipotent neural crest-derived stem

### Table 1. Sources and procedures of limbal epithelial stem cell transplantation to treat limbal stem cell deficiency.

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<th>Procedure</th>
<th>Result and highlight</th>
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<td>Human LESCs</td>
<td>Autologous and allogenic LESCs transplantation</td>
<td>Multiple LSCDs were treated. Structural and functional improvement in long-term follow-up reports.</td>
<td>[10–12,65–71]</td>
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<td>Human conjunctival epithelial cells</td>
<td>Autologous transplantation</td>
<td>Ex vivo procedure with certain therapeutic effect, but long-term evaluation is needed.</td>
<td>[73]</td>
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<tr>
<td>Human oral mucosal epithelial cell</td>
<td>Autologous transplantation</td>
<td>Ex vivo procedure, and visual acuity improvement in a majority of patients.</td>
<td>[74,75]</td>
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<td><strong>Animal experiments</strong></td>
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<tr>
<td>Human and rat mesenchymal stem cells</td>
<td>Differentiated to corneal epithelial cells, and transplantation</td>
<td>Showed therapeutic effects on rat model, but the effect may be associated with the inhibition of inflammation by MSCs.</td>
<td>[27,28]</td>
</tr>
<tr>
<td>Mouse hair follicle stem cells</td>
<td>Transdifferentiated to corneal epithelia like cells, and transplantation</td>
<td>The ocular surface was reconstructed on mice model.</td>
<td>[29,30]</td>
</tr>
<tr>
<td>Human dental pulp stem cells</td>
<td>Seeded on amniotic membrane for transplantation</td>
<td>Improved corneal transparency and reconstructed corneal epithelium but unclear about the mechanism of therapeutic effect.</td>
<td>[31]</td>
</tr>
<tr>
<td>Human umbilical cord lining stem cells</td>
<td>Seeded on amniotic membrane for allogenic transplantation</td>
<td>Express some LESCs markers, and showed the therapeutic effect on a rabbit injury model.</td>
<td>[32]</td>
</tr>
<tr>
<td>Skin epithelial stem cells</td>
<td>Transdifferentiation of skin epithelial stem cells to LESCs and transplantation</td>
<td>Defined the signal pathway and transcription factor that keep the corneal epithelial fate and proved the function of LESC-like cells transduced with Pax-6.</td>
<td>[33]</td>
</tr>
<tr>
<td>Mouse ESCs derived corneal epithelial like cells</td>
<td>Dissociated epithelial progenitors from mESCs transplantation</td>
<td>mESCs were induced to corneal epithelial progenitors. Corneal epithelium recovery within 24 h after transplantation.</td>
<td>[34,35]</td>
</tr>
</tbody>
</table>

ESC: Embryonic stem cell; LESC: Limbal epithelial stem cell; LSCD: Limbal stem cell deficiency; mESC mouse Embryonic Stem Cell; MSC: Mesenchymal Stem Cells.
A procedure where CECs were differentiated from mouse corneal stromal precursors by retinoic acid and activation of Wnt/(beta)catenin signaling was also reported, which confirmed the function of these CECs on a rabbit corneal disease model, and human CEC-like cells were acquired from differentiation of human corneal stromal precursors [52]. Corneal endothelial precursors were also found by sphere-forming assay [53] (Table 2). It is suggested that corneal endothelial stem cells may reside within the periphery of corneal endothelium and continually migrate centrifugally from the extreme periphery to the center of the corneal endothelium [54]. It seems that the peripheral cell populations have a higher density of precursors than the central part of the corneal endothelium [55].

Besides cell sources from cornea tissue, other stem cells, such as ESCs [56], cord blood mesenchymal stem cells [57], fetal bone-marrow-derived endothelial progenitor cells [58], adipose-derived stem cells [59] and neural crest cells [60] were differentiated to corneal endothelial-like cells. But iPSC-derived CEC-like cells have not been reported yet. Conditional medium or coculture system were applied in these stem cell differentiation protocols, meaning the specific molecule or signal pathway for CEC differentiation is still unclear. But, it looks like that CECs' differentiation need interplay with signals from other type of cells at the anterior eye segment [61], because not only CECs but also lens epithelial cells and CEC's biomimetic environment were utilized to drive these stem cells to CEC-like cells.

One of the crucial criteria to obtain CECs in vitro is to characterize cells with authentic markers of CECs. Although ZO-1, Na+-K+-ATPase and Occludin are used as the putative markers for CECs, they are also expressed by many other tissues, such as retinal pigment epithelial cells. Therefore, identification of additional specific markers for CECs is important to properly characterize the differentiation of CECs. Recently, our lab analyzed mRNA transcriptome in human fetal and adult CECs, and identified novel markers including Wnt5a, S100A4, S100A6 and IER3 as additional specific CEC markers in either fetal or adult stages [62]. In addition, Glypican-4 and CD200 were reported to distinguish human corneal endothelium from stromal fibroblasts [63]. The availability of these new markers would be helpful to characterize CEC-like cells derived from ESCs and iPSCs [64].

### Surgical procedures & therapeutic effects

The derivation of specific subtypes of corneal cells from stem cells is only the first step toward the treatment of corneal diseases. Indeed, it is equally important to develop good clinical procedures for delivering cells into corneal tissue. Below we review the recent progresses on the surgical procedures to deliver LESCs or CECs to treat LSCD and CEC deficiency.

#### Clinical application of LESCs

The clinical application of LESCs has a long history, especially for autologous LESC transplantation [60], which is now widely accepted to be the top

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**Table 2. Cell sources for corneal endothelial cell transplantation on animal models.**

<table>
<thead>
<tr>
<th>Cell sources</th>
<th>Procedure</th>
<th>Result and highlight</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult human CECs</td>
<td>In vitro culture</td>
<td>Showed limited in vitro proliferative capacity of adult CEC.</td>
<td>[40–45]</td>
</tr>
<tr>
<td></td>
<td>Establishment of CEC cell lines</td>
<td>CEC lines were generated by transduction of telomerase or Cdk4R24C (constitutively active mutant form of Cdk4) and CyclinD1 gene.</td>
<td>[46,47]</td>
</tr>
<tr>
<td>Fetal human CEC</td>
<td>Fetal CEC culture</td>
<td>Protocol of primary culture, passage and freezing fetal CECs.</td>
<td>[49]</td>
</tr>
<tr>
<td>Human and mice corneal precursors</td>
<td>Corneal stromal stem cells in vitro culture, differentiation and transplantation</td>
<td>There are precursors in corneal stroma, which could be induced to CEC-like cells, and showed function on rabbit model.</td>
<td>[50–52]</td>
</tr>
<tr>
<td></td>
<td>Culture of stem cells from human corneal endothelium.</td>
<td>There are precursors in corneal endothelium.</td>
<td>[53]</td>
</tr>
<tr>
<td>Human ESCs</td>
<td>Human ESCs differentiated to CECs and transplantation</td>
<td>ESCs were differentiated to CECs and showed function on rabbit corneal endothelium damage model for the first time.</td>
<td>[56]</td>
</tr>
<tr>
<td>Multi sources of multipotent stem cells</td>
<td>Rat neural crest cells, human umbilical cord blood mesenchymal stem cells, human fetal bone-marrow-derived endothelial progenitor cell differentiation.</td>
<td>These cells were induced to CEC-like cells and showed CECs' character in vitro.</td>
<td>[57–60]</td>
</tr>
</tbody>
</table>

CEC: Corneal endothelial cell; ESC: Embryonic stem cell.
choice for treating LSCD (65–70). For autologous LESC transplantation, patient’s contralateral eye’s LESCs were cultured on fibrin or amniotic membrane, then transplanted to the eye with LCSD (Table 1). Surgically, a small biopsy from patient’s contralateral eye is easy and safe, which can be operated in an outpatient clinic. In certain cases when autologous LESCs are not available from patients themselves, a biopsy of limbus from relatives or donor eye is an alternative to allow ex vivo expansion of LESCs. Because cultured cells do not have antigen presenting cells, transplantation of ex vivo expanded LESCs exhibits lower rejection rate compared with direct limbal transplantation. Finally, transplantation of ex vivo expansion of LESC has demonstrated the best satisfactory therapeutic effect on LSCD with minimal trauma to contralateral eye.

Two clinical studies have reported the outcome of LESC transplantation in more than several hundred patients over the period of a decade or longer [68,70]. According to these reports, over 70% patients’ corneal surfaces were functionally restored and kept stable, and some patients have been followed up over 10 years. Rama et al. reported that cultures containing more than 3.0% of ΔNp63α+ (an LESC marker) holoclones were successful in almost 80% of patients. If cultures contained 3.0% or less of ΔNp63α+ cells, the success rate drops to 11%. Rama et al. reported a way to improve the surgery success rate of LESC transplantation by enriching the p63+ cells in ex vivo culture. Other factors affecting the prognosis of LESC transplantation include severe tear film deficiency, uncontrolled inflammation and adnexal abnormalities [71,72].

In clinical practice, LSCD is also treated with other epithelial cells such as conjunctiva epithelium [73] and oral mucosal epithelium [74,75]. Some encouraging results were obtained from these techniques, but the number of clinical treatments is still very small and no superior clinical outcome was demonstrated when compared with the LESC transplantation. However, these two types of epithelia are easily acquired and can be applied to treat bilateral LCSD. Additionally, many other types of somatic stem cells are tested in clinical trial, or in preclinical animal models, including bone-marrow–derived mesenchymal stem cells [27,28], hair follicle stem cells [29], dental pulp stem cells [31], umbilical cord stem cells [32], and skin stem cells [33]. Finally, the potential use of pluripotent stem-cell–derived LESC-like cells are still at the stage of preclinical studies, awaiting for testing in small and large animal models of LSCD. With deepened understanding on LESC’s differentiation, more stem cell sources will available in clinical application in future. The indication for each type of source needs further research to identify.

CEC transplantation procedures

Due to the lack of donor eye, new procedures were developed for transplantations of CECs. Recently, Descemet’s stripping endothelial keratoplasty and DMEK [76] procedures showed better visual acuity improvement in clinical practice. But, there is no clinical report on ex vivo CEC treatment like LESCs, and cadavers are the only source for CECs transplantation. Because CECs have limited proliferative capacity in vivo, so the limited CEC source is still the major obstacle for CEC transplantation. Recently, some exciting progresses were reported in animal model experiments. CEC-like cells from ESCs were transplanted to rabbit CEC dysfunction model and showed therapeutic effect [56]. Mimura et al. published a rabbit model of CEC transplantation with cultured human CECs or CEC precursor cells [77,78]. Human CECs or CEC precursors were expanded ex vivo on collagen sheet, which were then transplanted into rabbit eyes that were stripped off CECs. After 3–4 weeks, they observed excellent therapeutic effects on corneal transparency in CEC transplanted eyes. The same strategy was applied in the monkey CEC deficiency model [79], with the monkey corneal edema showing recovery in clarity and decrease in overall corneal thickness. CEC-like cells derived from human umbilical cord blood mesenchymal stem cells [57], fetal bone-marrow–derived endothelial progenitor cells [58] or neural crest cells [60] were also tested, and exhibited modest therapeutic effect.

Although the above preclinical experiments indicate a promising strategy for clinical application, several concerns remain to be addressed, such as the safety of stem cells, therapeutic effect of the new procedure compared with DMEK and Descemet’s stripping endothelial keratoplasty with donated cornea. Meanwhile, the limited proliferative capacity of primary CECs is still a rate-limiting factor for ex vivo expansion, so the clinical potential is still uncertain. Nevertheless, because CEC transplantation is a relative immune-privileged site for corneal transplantation, if CEC-like cells from ESCs or iPSCs are successfully developed, CEC replacement therapy with sheet transplantation would be of great value in the treatment of CEC deficiency.

Conclusion

For eye diseases due to the deficiency of LESCs, autologous and allogenic limbal stem cells have been successfully used to treat LSCD patients in the past decade. Unilateral LSCD can be effectively treated by transplantation of autologous LESCs via ex vivo expansion. However, this procedure needs an biopsy from patient’s contralateral healthy eye, thus posing a potential risk for the healthy eye. For patients with
bilateral LSCD, allograft LESC transplantation is the option, but patients may face graft rejection in the long run. Therefore, LESCs derived from hESCs and hiPSCs would be very useful for clinical treatment of either unilateral or bilateral LSCD. At present, the efficacy and safety in the treatment of LSCD with mucosal and conjunctival epithelial cells remain to be proven by long-term follow-up of a large cohort of patients. In a parallel situation, patients with CEC diseases can be treated via transplantation of CEC sheet from donor eyes. Because pluripotent stem cells can be induced into functional CECs or CEC-like cells in vitro, we expect that stem-cell-derived CECs would be available for treating CEC deficiency in the near future.

Future perspective
With increased understanding of the molecular events underlying corneal epithelial and endothelial lineage differentiation, pluripotent and somatic stem cells would be effectively induced to differentiate into LESC and CECs. Future clinical trials would also determine the concern of the immune rejection, the efficacy and safety of either hESC- or hiPSC-derived LESC and CECs in vivo. Considering the advantage of manufacturing a large quantity of clinical-grade hESCs or hiPSCs for cell differentiation, we believe that a bank of human ESC- and iPSC-derived LESCs would provide a most useful and economic cell source for treating LSCD patients who cannot pursue the ex vivo expansion of autologous LESCs. Finally, although stem-cell-derived LESC and CECs have been tested for the efficacy and safety in animal models of LSCD and CEC deficiency, only rigorous clinical trials and long-term follow-up of patients would eventually vindicate stem-cell-based therapy for treating patients with corneal epithelial and endothelial diseases.

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Executive summary
Patients with corneal epithelial & endothelial diseases require stem-cell-based therapy
- Corneal transplantation is a cure to many severe corneal diseases. However, the donor shortage in developing countries greatly hindered the treatment of corneal diseases worldwide.
- Limbal stem cell deficiency (LSCD) is a group of diseases with defects in limbal epithelial stem cells (LESCs) that can be treated by transplantation of either ex vivo expansion of LESC or stem-cell-derived LESC.
- Due to the paucity of proliferation capacity of adult corneal endothelial cells (CECs) ex vivo, CEC density decreases with age. CEC deficiency can be caused by degenerative conditions, trauma and intraocular surgery procedure.

Regenerative medicine for the treatment of LSCD & CEC deficiency
- Autologous ex vivo expansion of LESC and transplantation is the first choice for unilateral LSCD with a successful long-term follow-up record.
- LESC derived from a variety of somatic and pluripotent stem cells are promising for the treatment of both unilateral and bilateral LSCD patients.
- Current treatment of CEC deficiency is limited to CEC sheet transplantation or cornea transplantation.
- Limited success is achieved in the differentiation of stem cells into CECs.
- With the improvement of CEC transplantation procedure such as Descemet’s membrane endothelial keratoplasty, stem-cell-derived CEC transplantation holds a great promise for treating CEC deficiency diseases in the near future.

References
Papers of special note have been highlighted as:
* of interest; ** of considerable interest


This is the first paper about human pluripotent Stem Cells derived corneal endothelial cell like cells that showed in vivo function in rabbit experiments.


Transcriptome analysis on corneal endothelial cells (CECs) provided valuable information on CEC’s development and identified the CECs’ novel functional markers.


