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From Synthetic Biology to Human Therapy: Engineered Mammalian Cells

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Abstract

Mammalian synthetic biology has evolved to become a key driver of biomedical innovation in the area of cell therapy. Advances in receptor engineering, immunotherapy and cell implants promise new treatment options for complex diseases. Synthetic receptors have already found applications in cellular immunotherapy for cancer treatment, and are being introduced into the field of cell implants. Here, we discuss prospects for the next generation of engineered mammalian cells for human therapy, highlighting selected recent studies.

Keywords: Antigen Receptor, Bioengineering, Cell Engineering, Cell Therapy, Cytokine Receptor, Genetic Engineering, Immunotherapy, Protein Engineering, Synthetic Biology

Highlights

- Engineered receptors reroute tumor antigens to therapeutic responses.
- Translating soluble markers of the tumor microenvironment into immunostimulatory cell behavior.
- Targeting infectious diseases by pathogen sensing cell implants.
- Establishing novel cell types for more effective cell therapy.

Introduction

Cell therapy is rapidly emerging as major technological innovation in medical science. In contrast to traditional treatments with small-molecule drugs, cell therapy requires the delivery of living therapeutic cells to the patient. In some cases, wild-type cells already meet the requirements of a desired application, as exemplified by mesenchymal stem cells (MSC), which can be isolated from the patient, amplified *ex vivo* and reinjected to treat tissue injury and immune disorders [1]. In many cases, however, no native cell types exhibit the desired therapeutic phenotype. Medical efficacy can then still be achieved by genetic engineering,

either by equipping cells with novel functions or by enhancing or redirecting the natural abilities of specialized cell types [2•, 3] Typically, therapeutic cells need to: 1. sense disease associated markers, 2. process the information, and 3. respond with a desired function (Figure 1a). The cells therefore work as smart drugs that act situation-dependently. This approach excels in the treatment of complex diseases such as cancer or metabolic disorders, where small-molecular drugs or antibodies have limitations [4, 5••]

In particular, the field of receptor engineering has made great progress within the last two years and provides predictable input-output modules for various applications [6]. For example, engineered cells can be used in therapeutic cell implants that sense disease-associated biomarkers and secrete therapeutic peptides in response [4] (Figure 1b). Another major application of engineered cells is in the field of immunotherapy, where a patient's own immune cells are genetically modified for enhanced oncolytic activity. This technology requires the isolation of autologous immune cells, *ex vivo* expansion, genetic engineering and redelivery into the patient (Figure 1c) [5••]. Engineering cells for cell therapy is an immediately clinically relevant application of mammalian synthetic biology, and recent advances point towards novel treatment options for various diseases. In the following paragraphs we highlight current developments in receptor engineering, cell implants and cellular immunotherapy, and discuss how concepts from these fields could be combined in future research.

Receptors for cell-surface tumor antigens

Synthetic receptors that recognize surface antigens require direct cell-cell contact for activation. Therefore, they are especially attractive for focused lysis of target cells, as any off-target lysis would likely result in detrimental side effects.

The recent regulatory approval of the first CAR (chimeric antigen receptor)-T cell therapies and several ongoing clinical trials are prime examples of the clinical potential of mammalian synthetic biology [5••,7]. The CAR utilizes an antibody fragment (single chain variable fragment; scFv) to recognize tumor antigens displayed on cancer cells, and scFv binding activates intracellular signaling domains for oncolytic activity and T-cell proliferation (Figure 2a). Current research is focused on enhancing CAR-T cell activity against solid tumors and on reducing side effects. Especially the immunosuppressive tumor microenvironment has gathered attention as a major challenge that needs to be addressed by the next generation of CAR-T cells [8]. The large number of ongoing clinical trials illustrates the relevance of the field, and many excellent reviews discuss current strategies in detail [5••, 9, 10, 11]

Synthetic Notch (SynNotch) receptors consist of an scFv fused to an engineered Notch

receptor. Cell-contact-dependent antigen recognition is considered to pull up the Notch transmembrane helix, exposing a cleavage site to an intramembrane protease. Cleavage releases a membrane-coupled intracellular transcription factor that relocates to the nucleus and activates transgene expression [12,13] (Figure 2b). Expressing CARs downstream of SynNotch enables AND-gate logic for cell-surface antigens, which might reduce side effects by enhancing specificity [14]. Another exciting application of SynNotch receptors is organization of microtissue formation [15**] in synthetic morphology [16]. SynNotch receptors have proven to be a robust tool for inducing orthogonal transgene expression in response to cell-surface antigens, and due to the ubiquitous nature of cell contact dependent signaling in different branches of biology, this methodology is likely to find applications in various fields of research.

Cytokine receptor signaling was rendered cell-contact-dependent by co-expressing an inhibitory receptor that is spatially removed from the cytokine receptor complexes upon target-cell binding (Figure 2c). This activation mechanism is reminiscent of TCR (T-cell receptor) activation, but was functional in non-immune cells, such as a mesenchymal stem cell (MSC) line. The engineered cells recognized cancer cells *in vitro* and in response produced an enzyme for pro-drug conversion to mediate cytotoxic effects [17•]. A similar mechanism was used to remove a constitutively active RhoA protein from the cell-cell interface between the engineered therapeutic cell and a marker-expressing target cell. The resulting RhoA polarization drives cell invasion and/or cell fusion of the RhoA-expressing cell with the target cell [18]. Eventually, this mechanism might enable targeted delivery of complete genetic circuits for sophisticated responses in cancer therapy.

Receptors for soluble ligands

While cell-contact-dependent receptors mostly focus on direct lysis of cancer cells, receptors for soluble ligands enable medium to long-range communication between cell populations. For example, sensing tumor-associated biomarker concentrations and linking them to an immunostimulatory response is a promising strategy to transform the tumor microenvironment and enhance the effectiveness of immunotherapy.

MESA (Modular Extracellular Sensor Architecture) receptors are based on antigen-mediated dimerization of two single-pass transmembrane helices that are intracellularly fused to either a transcription factor or a TEV (tobacco etch virus) protease [19,20]. Antigen-mediated dimerization of the receptor chains increases the likelihood that the TF will be cleaved and released from the plasma membrane to drive transgene expression (Figure 2d). This system was used to sense VEGF (vascular endothelial growth factor) and secrete IL-2

(interleukin 2) in response; VEGF is a dimeric signaling protein that is overexpressed in many cancers and IL-2 is an important regulator of T-cell responses. This study provides a proof of concept for a rationally designed synthetic receptor. Improvements in sensitivity and signal-to-noise ratio will still be required to bring this technology closer to clinical application.

Chimeric receptors combine ligand-binding extracellular domains (ECDs) with signal-transducing intracellular domains (ICDs) from different receptors. In many studies, cytokine receptors are exploited for this purpose, as their modular design often tolerates recombination of receptor domains. For instance, combining the ECD of IL-4R (interleukin-4 receptor) with the ICD of the immunostimulatory IL-7R resulted in receptors that sense IL-4 but respond via IL-7R-specific signaling pathways, including STAT5 or PI3K/AKT signaling which are involved in activating T-cells [21**] (Figure 2e). This receptor was used to enhance CAR-T cell function in the tumor microenvironment, but the principles of receptor engineering applied in this study are not limited to one specific application. Chimeric receptors have great potential for controlling cell behavior in response to various signaling molecules in many applications, including cell therapy.

The application of chimeric receptors is not limited to immunostimulation; they can be combined with other engineered proteins to obtain diverse effects, such as engineering cells to seek out and lyse IL-6-secreting senescent cells. This was achieved by fusing IL-6R ECD to VEGFR ICD to control an engineered RhoA GTPase. Activation of VEGFR ICDs leads to a polarized increase in intracellular Ca^{2+} concentration. Ca^{2+} activates the engineered RhoA GTPase, causing directed cell migration along the IL-6 gradient [22•, 23, 24] (Figure 2f). Most types of cell therapy rely on proximity between therapeutic and target cells. Therefore, development of tools for directed cell migration towards disease-associated cell populations is a highly promising approach. As shown, this strategy can find applications beyond cancer therapy, but while VEGFR signaling is probably not immediately applicable for engineering cancer-targeting immune cells, novel receptors for tumor targeted cell migration might contribute to the ongoing efforts of seeking out metastases or enhancing cell infiltration into solid tumors.

Chimeric receptors based on the EpoR (erythropoietin receptor) ECD fused to the signaling domain of IL-6R were first utilized to implement antigen-mediated growth responses in various blood hematopoietic cell lines [25,26]. Based on this system, a platform of motif-engineered receptors was developed that signals only via rationally assembled motifs to obtain enhanced control over the activated signaling pathways, such as activation of Shc and STAT3 or STAT5. This system was used to investigate the effects of pathway cooperation on

hematopoietic stem cell proliferation [27] (Figure 2g). Apart from direct applications in cell proliferation control, these studies highlight the modularity of cytokine receptors, which is useful for generating novel synthetic receptors for therapeutic applications.

Using a similar core architecture as the chimeric receptors based on EpoR/IL-6R fusions, we recently developed the GEMS (generalized extracellular molecule sensor) platform for antigen-mediated activation of the intracellular domains of IL-6R, VEGFR and FGFR (fibroblast growth factor receptor). The design tolerates various affinity domains in the same framework, as was shown by generating receptors for input molecules having a wide range of molecular weight. For example, we engineered GEMS receptors for the soluble cancer antigen PSA (prostate specific antigen). Signaling was induced by PSA concentrations in the diagnostically relevant concentration range, and this approach might thus be another step towards engineering therapeutic cells with predictable responses in the tumor microenvironment [28**] (Figure 2h). The first *in vivo* application of the GEMS platform is the C-STAR (caffeine-stimulated advanced regulators) system. C-STAR consists of cells equipped with an engineered caffeine receptor rerouted to drive shGLP-1 (synthetic human glucagon-like peptide 1) expression. Cell implants containing these cells successfully counteracted experimental diabetes in response to coffee consumption, providing a proof of principle for integrating therapy with lifestyle, which might improve patient compliance [29] (Figure 2i).

Some of the systems, such as SynNotch and MESA, are unlikely to interfere with mammalian signaling and are therefore considered orthogonal, whereas other systems such as CAR-T or GEMS rely on endogenous signaling pathways. Both strategies have advantages, depending on the application. For example, expression of single transgenes might benefit from orthogonal signaling, as inadvertent expression is less likely. Endogenous signaling on the other hand allows for more complex responses, as seen in CAR-T. Also, evolved signaling cascades as used in GEMS generally perform with higher signal-to-noise ratios than other current rationally designed systems.

Smart cell implants

Encapsulating and implanting engineered cells minimizes possible side effects of cell therapy [30]. The pores of commonly used capsules are permeable to proteins and nutrients, but are too small for cells to penetrate. Encapsulation therefore protects both the implanted cells from the immune system and the host from the implanted cells.

Implantation of cells that sense biomarkers and in response produce therapeutic peptides has been investigated as a means of treating diseases that require constant monitoring

of biomarkers and dose-adjusted delivery of therapeutics. Ideally, treating the disease results in reduced biomarker concentrations, providing a closed loop with self-adjusting drug dosage [4] (Figure 3a). Such closed-loop systems are best exemplified by cell implants for treating experimental diabetes, consisting of cells that detect glucose and secrete shGLP-1 in response [31]. Major advances in smart cell implants were made with cell implants targeting infection with biofilm-forming bacteria. One system utilizes the ability of TLRs (Toll like receptors) to recognize bacterial biomarkers, and reroutes TLR signaling to trigger the expression of an antibacterial peptide. The resulting cell implants (named InfectPro) successfully protected mice from MRSA (methicillin-resistant *Staphylococcus aureus*) infection [32•] (Figure 3b). Interfering with bacterial quorum sensing is another strategy for reducing biofilm formation. For that purpose, a biosynthetic pathway was engineered for production of the autoinducer AI-2 in mammalian cells and placed under control of a bacterial biomarker sensing GPCR (G protein-coupled receptor) FPR1. Cells equipped with this system responded to bacterial infection with AI-2 production and effectively interfered with biofilm formation [33•] (Figure 3c). In contrast to AI-2, other autoinducers, such as *Pseudomonas* autoinducer (PAI) 1 and 2 promote biofilm formation. A nuclear autoinducer sensor was engineered to sense PAI-1 and in response activate expression of a combination of biofilm- and autoinducer-degrading enzymes [34] (Figure 3d). These studies showcase the effectiveness of therapeutics produced *in vivo*, even though the same molecules might be unsuited for traditional delivery routes due to low serum half-life or poor oral bioavailability. Delivering such drugs with cell implants might become a viable alternative to antibiotics for combatting the growing problem of drug-resistant pathogens.

Cell lines for cell implants

Most cell implants have been characterized with HEK293 (human embryonic kidney) cells, as this cell type is well adapted for highly efficient transfection, easy culture, and high secretion levels. Encapsulated HEK293 cells remain functional in terms of continuous, controlled production of therapeutic peptides for up to three weeks [35]. However, advancing this technology towards clinical application will change the requirements on the cell type. In a clinical setup, cells need to persist for a long time within the patient and keep performing consistently. Additionally, the secretome of such cells should be compatible with the desired application, while avoiding adverse effects [36, 37] Engineering autologous cells might help reduce possible host vs implant reactions. However, such cells might also be more likely to form malignancies if they escape the implant. It can be speculated that large-scale Cas9-

mediated knockouts would be applicable for customizing the secretomes of autologous cells or cell lines to obtain improved clinical performance.

Transposase-mediated genomic integration [38,39] accelerates the generation of permanently engineered cell lines for implants. This approach also simplifies the use of other cell lines and might ease the transition to cell types that are approved for human therapy [29,40,41].

Engineering immune cells

Immune cells are responsible for major defensive functions against infectious diseases and cancer. They have the ability to migrate to disease loci, secrete immunomodulatory molecules, and lyse target cells. Engineering of these features for novel applications is a highly promising direction of ongoing research.

Most research in the area of immune cell engineering focuses on T-cells, as they are comparatively easy to obtain, genetically engineer and expand *ex vivo* [42]. Crispr/Cas9 has been used to further improve cell behavior in therapeutic settings. For example, knocking out PD1, which is an important negative regulator of T-cell responses, enhances CAR-T cell activity in the immunosuppressive tumor microenvironment [43••]. Other studies show that integrating CARs into the native genomic TCR locus prevents premature T-cell exhaustion, and that knocking out endogenous TCRs improves performance of transgenic TCRs [44••, 45] (Figure 4a). In addition to having immediate applications in therapy, these results demonstrate that cell functions are heavily influenced by various cell-specific regulatory mechanisms, and that engineered proteins cannot be viewed as isolated from the host cell.

Dendritic cells and natural killer (NK) cells are two other major cell types with roles in immune defense against cancer. NK-cells equipped with CARs for directed tumor cell lysis are emerging as an alternative to CAR-T cells. Clinical translatability of engineered NK-cells lags behind that of T-cells due to bottlenecks in obtaining sufficient cell numbers and in genetically engineering these cells. Genetically engineering iPS (induced pluripotent stem) cells with CAR receptors and afterwards differentiating them into NK cells is therefore a major advance towards generating sufficient numbers of functional CAR-NK cells [46]. An optimized CAR receptor with NK-specific costimulatory domains further enhanced the efficiency of NK-directed tumor lysis [47••] (Figure 4b).

Dendritic cells are key coordinators of immune responses by displaying antigens and stimulating effector cells. Dendritic cells loaded with cancer antigens are already used to stimulate Th1 responses for cancer therapy. Dysregulated dendritic cells in the tumor

microenvironment are a promising target for ongoing efforts to further improve this technology (Figure 4c) [48].

Macrophages have typically been less effective in cancer treatments, due to their sensitivity to the inhibitory tumor microenvironment. A new study, however, shows that blocking inhibitory receptors on the macrophages combined with pre-loading tumor-specific antibodies can be highly effective to overcome this issue [49] (Figure 4d). Drug-loaded nanoparticles are another promising route for targeting tumor-associated macrophages [50-52].

The increasing number of synthetic receptors provides many opportunities to engineer cell types beyond CAR-T cells for uses in immunotherapy. Expanding the repertoire of immune cells susceptible to engineering for immunotherapy opens up the possibility of combining multiple cell types to obtain concerted anti-tumor effects. For example, CD4⁺ T cells are critical for efficient anti-tumor responses [53] and the interplay of NK cells with dendritic cells is well documented [54]. Engineering these cells for optimized behavior in the tumor microenvironment is a promising option for enhancing immunotherapy that might be further exploited in the future.

Outlook and challenges

Mammalian synthetic biology has reached the point where a multitude of proof-of-concept studies has provided us with a large toolbox for cell engineering and a sufficient understanding of the underlying biology to start realizing medical applications. Cancer treatment with CAR-T cells is the pioneering application. Combining different immunotherapies is a highly promising direction for future research [9]. Integrating different engineered cell types and optimizing intercellular communication by means of engineered cytokine receptors [21•, 28••] would add another layer of complexity, but might improve immune cell function [55,56]. CRISPR/Cas9 could be useful in further customizing cellular phenotypes, for example by knockouts of endogenous receptors or cell-surface markers [43••], by customizing the secretome [36], or by placing transgene expression cassettes under the control of endogenous regulatory mechanisms [44••]. iPSCs that can be engineered, expanded and differentiated into target cells provide another highly promising avenue to help overcoming current bottlenecks in creating therapeutic cells [46,57]. The topics discussed in this review should therefore not be viewed simply as mutually exclusive approaches to treat separate diseases, but as strategies that can complement each other, offering synergistic effects in novel applications.

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Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

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Figure Legends

Figure 1 | Engineering cells for therapy. (a) Therapeutic cells act as smart drugs by adjusting cellular responses according to environmental cues. Inputs and outputs are highly variable. (b) Typical examples of inputs and outputs include sensing of disease-associated molecules and secretion of therapeutic peptides. Cells with such behavior can be encapsulated and implanted in patients to treat disease. (c) Some cell types can be isolated from patients, expanded and transduced ex vivo, and redelivered afterwards.

Figure 2 | Engineering synthetic receptors. (a) Chimeric antigen receptors activate endogenous signaling cascades for T-cell responses upon scFv (single chain variable fragment)-mediated binding of membrane-displayed antigens. The intracellular domains (ICDs) are optimized for effective T-cell activation in response to cancer markers. (b) SynNotch receptors cleave a custom transcription factor from the membrane in response to scFv-mediated binding of the membrane-displayed target. The liberated transcription factor relocates to the nucleus to activate transgene expression. (c) IL-4R (interleukin 4 receptor) and IL-13R heterodimers engineered for cell-contact-dependent signaling. scFv-mediated receptor binding to the membrane-displayed target antigen decreases the distance between the membranes of the sender cell and receiver cell, which segregates the bulky extracellular domain (ECD) of CD43 from the cell-cell interface. CD43 ECD is fused to the inhibitory ICD of CD45. Segregating CD45 domains from the receptor dimers restores cytokine signaling. (d) Antigen-mediated dimerization of MESA receptor chains increases the probability of custom transcription factor cleavage. The liberated transcription factor relocates to the nucleus to activate transgene expression. (e) Chimeric cytokine receptor consisting of IL-4R ECD and IL-7R ICD. The chimeric receptor heterodimerizes with endogenous IL-2rg in response to IL-4, but activates IL-7-specific signaling pathways. (f) Chimeric cytokine receptor consisting of IL-6R ECD and VEGFR ICD. The receptor activates VEGFR-specific Ca^{2+} signaling in response to IL-6. The polar differences in intracellular Ca^{2+} were translated into directed cell migration via an engineered RhoA GTPase. (g) Motif-engineered receptors for antigen (shown here: fluoresceinated albumin)-mediated stimulation of cell growth. The ICD was rationally designed

for enhanced control over activated signaling pathways. (h) GEMS receptors for sensing various inputs to activate multiple endogenous signaling pathways. This example shows GEMS engineered to activate transgene expression in response to the tumor marker PSA. (i) C-STAR utilizes GEMS equipped with caffeine antibodies to reroute caffeine stimulation to transgenic GLP-1 expression for treating experimental diabetes.

Figure 3 | Closed-loop cell implants. (a) Smart cell implants boost therapeutic peptide production in response to increases in biomarker concentration. Closed-loop adjustment of drug dosage happens when disease treatment in turn reduces biomarker concentration. (b) The InfectPro system reroutes TLR (toll like receptor) signaling to expression of antibacterial peptides. (c) Activity of an engineered biosynthesis pathway for autoinducer AI-2 production placed under control of the GPCR (G protein-coupled receptor) FPR1 that signals in response to bacterial biomarkers. Interference with bacterial autoinducer systems controls biofilm formation. (c) Expression of a combination of biofilm- and autoinducer-degrading enzymes placed under the control of a nuclear sensor of biofilm-promoting autoinducer PAI-1 of *Pseudomonas aeruginosa*.

Figure 4 | Engineered immune cells. (a) T-Cells equipped with engineered CARs or TCRs for cancer-antigen-dependent T-cell activation. Expression of CARs from the endogenous genomic TCR locus and knockout of the inhibitory receptor PD1 improve performance. (b) Novel NK-CARs enhance the function of CAR-NK-Cells. (c) Dendritic cells loaded with cancer antigens enhance endogenous immune responses against cancer. (d) Macrophages pre-loaded with tumor-targeting antibodies for cancer cell recognition and phagocytosis. Simultaneous blocking of inhibitory receptors enhances effectiveness in the tumor microenvironment.

Figure 1

Figure 2

Figure 3

Figure 4