DRUG DELIVERY
2018, VOL. 25, NO. 1, 1234–1257
https://doi.org/10.1080/10717544.2018.1474964

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REVIEW ARTICLE

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Delivering CRISPR: a review of the challenges and approaches

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ABSTRACT

Gene therapy has long held promise to correct a variety of human diseases and defects. Discovery of the Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR), the mechanism of the CRISPR-based prokaryotic adaptive immune system (CRISPR-associated system, Cas), and its repurposing into a potent gene editing tool has revolutionized the field of molecular biology and generated excitement for new and improved gene therapies. Additionally, the simplicity and flexibility of the CRISPR/Cas9 site-specific nuclease system has led to its widespread use in many biological research areas including development of model cell lines, discovering mechanisms of disease, identifying disease targets, development of transgene animals and plants, and transcriptional modulation. In this review, we present the brief history and basic mechanisms of the CRISPR/Cas9 system and its predecessors (ZFNs and TALENs), lessons learned from past human gene therapy efforts, and recent modifications of CRISPR/Cas9 to provide functions beyond gene editing. We introduce several factors that influence CRISPR/Cas9 efficacy which must be addressed before effective *in vivo* human gene therapy can be realized. The focus then turns to the most difficult barrier to potential *in vivo* use of CRISPR/Cas9, delivery. We detail the various cargos and delivery vehicles reported for CRISPR/Cas9, including physical delivery methods (e.g. microinjection; electroporation), viral delivery methods (e.g. adeno-associated virus (AAV); full-sized adenovirus and lentivirus), and non-viral delivery methods (e.g. liposomes; polyplexes; gold particles), and discuss their relative merits. We also examine several technologies that, while not currently reported for CRISPR/Cas9 delivery, appear to have promise in this field. The therapeutic potential of CRISPR/Cas9 is vast and will only increase as the technology and its delivery improves.

ARTICLE HISTORY
Received 12 February 2018
Revised 3 May 2018
Accepted 7 May 2018

KEYWORDS
CRISPR; Cas9; history; overview; review; drug delivery; prospective

Introduction

Discovery of the Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR) (Ishino et al., 1987; Mojica et al., 1993; van Soolingen et al., 1993), their function as part of an adaptive prokaryotic immune system (CRISPR-associated system, Cas) (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005; van der Oost et al., 2009), and subsequent development into a genomic editing tool (Jinek et al., 2012; Cho et al., 2013; Cong et al., 2013; Mali et al., 2013), has revolutionized the field of molecular biology. Much of this enthusiasm centers on the clinical potential of CRISPR/Cas9 for treating human disease and editing the human genome. However, the simplicity and specificity with which CRISPR/Cas9 can edit DNA is changing the pace of biological research in many areas, including identifying and understanding mechanisms of genetic diseases (Findlay et al., 2014; Gilbert et al., 2014; Zhou et al., 2014; Konermann et al., 2015), validating disease targets (Shalem et al., 2014; Wang et al., 2014), developing animal disease models (Wang et al., 2013; Yang et al., 2013), facilitating genetic engineering in plants (Raitskin & Patron, 2016; Zhang et al., 2016, 2017), and allowing for more thorough epigenetic studies (Yao et al., 2015; Vora et al., 2016). This broad impact of the CRISPR/Cas9 gene editing tool has led to over 6000 research publications since its development five years ago.

Gene therapy may greatly benefit from CRISPR/Cas9 technology. To date, over 3000 genes have been associated with disease-causing mutations (Cox et al., 2015). Early efforts to correct disease-causing genetic mutations in humans, although generally successful, were tainted by several tragedies. Perhaps the most well-known early gene therapy trial involved two studies from France (Hacein-Bey-Abina et al., 2002; Hacein-Bey-Abina et al., 2010) and the UK (Gaspar et al., 2004; Gaspar et al., 2011) of children suffering from X-linked severe combined immunodeficiency (SCID X-1). Of the 20 patents participating in the trial, 17 were successfully and stably cured (Cavazzana et al., 2016). However, five children subsequently developed T-cell leukemia, with one child dying from chemotherapy-refractory leukemia. In all cases of leukemia, the SCID X-1 correcting gene had inserted into the patient genome within or near tumor-promoting genes and caused transcriptional activation (Check, 2002; Kaiser, 2003; Thomas et al., 2003). In another tragedy, an 18-year-old male suffering from a partial deficiency of ornithine transcarbamylase (OTC) died after developing a massive inflammatory response to the genetic cargo delivery vehicle, an adenovirus

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vector, four hours after receiving the treatment (Marshall, 1999; Authors, 2002).

It is important to note that both tragedies stemmed from the therapeutic delivery method (LaFountaine et al., 2015). In the case of SCID X-1, the correcting gene construct was non-specifically inserted into the genome; in the case of partial OTC, the viral vector induced a severe immune response. It is therefore critical that gene therapy technologies allow for highly specific editing of the genome to reduce the risk of undesired mutagenesis, and that the delivery vehicle allows for safe and efficient transport to the target.

In just one decade after these tragedies, great progress has been made in advancing gene therapy technologies, leading to renewed enthusiasm in the promise of broad-spectrum treatment of genetic diseases. Advances include the discovery and development of site-specific nucleases for gene editing: zinc finger nucleases (ZFNs) (Bibikova et al., 2002), transcription activator-like effector nucleases (TALENs) (Christian et al., 2010), and CRISPR/Cas9. Advances further include tools for delivery of the cargo to targeted cells for genetic editing, both *ex vivo* and *in vivo*. Careful consideration and development of both the gene editing tool and the delivery mechanism will be required if the full potential of therapeutic gene editing is to be realized. This review will briefly introduce ZFNs and TALENs, then provide an in-depth description of the CRISPR/Cas9 system, including recent advances and modifications to the technology, and factors affecting system performance. This will be followed by a comprehensive synopsis of existing CRISPR/Cas9 delivery methods, their potential and challenges in delivering CRISPR, and recently reported promising candidates for delivery of gene editing systems.

Gene editing

At the core, gene gain/loss-of-function therapy comprises (1) the generation of double-stranded breaks (DSBs) in defined regions of the genome, (2) correction of the defective endogenous genes or introduction of exogenous genes, and (3) DSB repair. DSBs in eukaryotes are repaired by one of two endogenous repair mechanisms: non-homologous end joining (NHEJ) or homology-directed repair (HDR). In NHEJ, protein factors re-ligate the broken DNA strand either directly or by including nucleotide insertions or deletions (indels) (Hefferin & Tomkinson, 2005). This process occurs without a homologous DNA template, regularly leading to mutations and deletions in the repaired strand (Bibikova et al., 2002), as shown in Figure 1(A). NHEJ is, therefore, characterized as error-prone. NHEJ can occur at any phase of the cell cycle and is the primary cellular DSB repair mechanism. In contrast, HDR uses a homologous repair template to precisely repair the DSB (Capecchi, 1989; Takata et al., 1998) (see Figure 1(B)). HDR typically occurs in late S- or G_2-phase, when a sister chromatid can serve as the repair template. In general, the incidence of HDR for DSB repair is extremely low compared with NHEJ, at least in instances where both pathways are equally available to an organism. Given the significant gene editing enabled by HDR, development of methods to

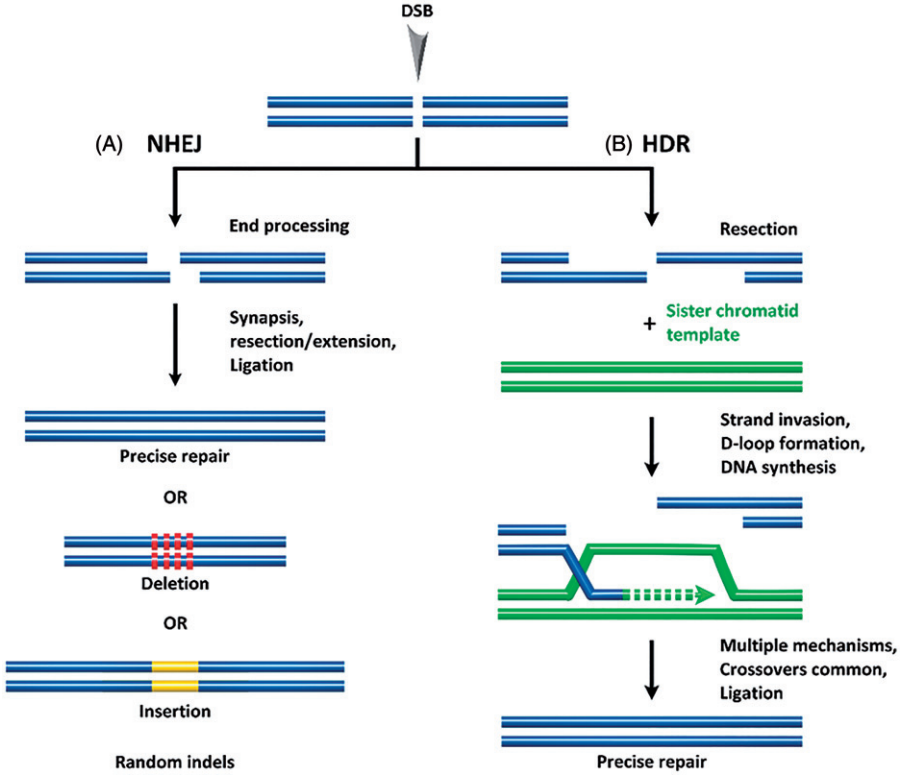

Figure 1. Following formation of a double stranded break (DSB), endogenous DNA repair can occur by (A) non-homologous end joining (NHEJ) resulting in random indels, or by (B) homology-directed repair (HDR) which uses a template DNA strand for precise repair.

increase the incidence/efficiency of HDR for gene editing with site-specific nucleases is an active field of research.

The use of site-specific nucleases and NHEJ or HDR generally results in one of four gene editing products. Shown in Figure 2, these include gene knockout, deletion, correction, or addition. The error-prone character of NHEJ can be exploited to introduce indels and frameshifts into the coding regions of a gene. This knocks the gene out (Figure 2(A)) via nonsense-mediated decay of the mRNA transcript. In gene deletion (Figure 2(B)), paired nucleases excise regions of the coding gene, resulting in premature truncation and knockout of the protein in a manner more generally efficient than introducing frameshifts. Both gene correction (Figure 2(C)) and gene addition (Figure 2(D)) require an exogenous DNA template that can be introduced as either single-stranded (Radecke et al., 2010; Chen et al., 2011; Soldner et al., 2011) or double-stranded DNA (Rouet et al., 1994). The DNA template contains homologous sequence arms that flank the region containing the desired mutation or gene cassette.

Site-specific nucleases

To address challenges with non-specific insertion, provide greater fidelity, and assist in more precise gene editing, programable nucleases have been developed.

Zinc finger nucleases (ZFNs)

In 2002, the first sequence-specific nucleases, termed zinc finger nucleases (ZFNs), were reported by Bibikova et al. (2002, 2003). ZFNs are a fusion protein of Cys2-His2 zinc finger proteins (ZFPs) and a non-specific DNA restriction enzyme derived from *Fok*I endonucleases, as shown in Figure 3(A). ZFPs are common in eukaryotic cells and are associated with transcriptional regulation and protein–protein interactions (Wolfe et al., 2000; Urnov et al., 2010). For an in-depth review on specific ZFN function, see the review by Carroll (2011).

Challenges with ZFNs include design and engineering of the ZFP for high-affinity binding of the desired sequence, which can prove non-trivial (Ramirez et al., 2008). Also, not all sequences are available for ZFP binding, so site selection is limited. Using open-source ZFP domains, sites could be targeted only every 200-bps in a random DNA sequence (Gupta & Musunuru, 2014). This may not be a concern if the objective is gene knockout or deletion; however, this may be an obstacle if the objective is a gene correction or addition product. Another significant challenge is off-target cutting (Gabriel et al., 2011; Pattanayak et al., 2011). ZFN design improvements addressing off-target concerns have included ZFNs that work in pairs, with each pair recognizing two sequences that flank the target cleavage site. One ZFN binds the forward strand, and the second ZFN binds the reverse strand, increasing the total number of recognized bps to between 18 and 36. Further, *Fok*I domains that are obligate heterodimers with opposite charge have been fused to ZFPs such that only properly paired ZFNs will result in *Fok*I dimerization/activity and the formation of a DSB (see Figure 3(A)) (Miller et al., 2007; Doyon et al., 2011).

Transcription activator-like effector nucleases (TALENS)

Four years following the development of ZFNs, a new class of natural DNA-binding proteins was discovered in the plant pathogenic bacteria *Xanthomonas sp.* (Zhang et al., 2013). The proteins, termed transcription activator-like effectors (TALEs), contain 33–35 amino acid repeats that flank a central DNA binding region (amino acids 12 and 13). This DNA binding region, known as the repeat variable di-residues (RVDs), specifically binds the DNA (Christian et al., 2010; Miller et al., 2011) as shown in Figure 3(B). Shortly after the discovery of TALEs, TALE nucleases (TALENs) were developed that, like ZFNs, are a fusion protein comprised of a TALE and a *Fok*I nuclease (Christian et al., 2010; Miller et al., 2011; Li et al., 2011; Reyon et al., 2012). For an in-depth TALEN function review, see Joung & Sander (2013).

Unlike ZFNs, design and engineering of TALENs is much simpler and can be done in a shorter time (Cermak et al., 2011; Reyon et al., 2012). TALENs are also not as limited as ZFNs in target site selection due to the 1:1 TALE–DNA binding (Zhu et al., 2013). While off-target cutting remains a concern, TALENs have been shown in one side-by-side comparison study to be more specific and less cytotoxic than ZFNs (Mussolino et al., 2014). TALENs, however, are substantially larger than ZFNs, requiring 3 kb of cDNA encoding for one TALEN versus just 1 kb for a single ZFN. This makes delivery of a pair of TALENs more challenging than a pair of ZFNs

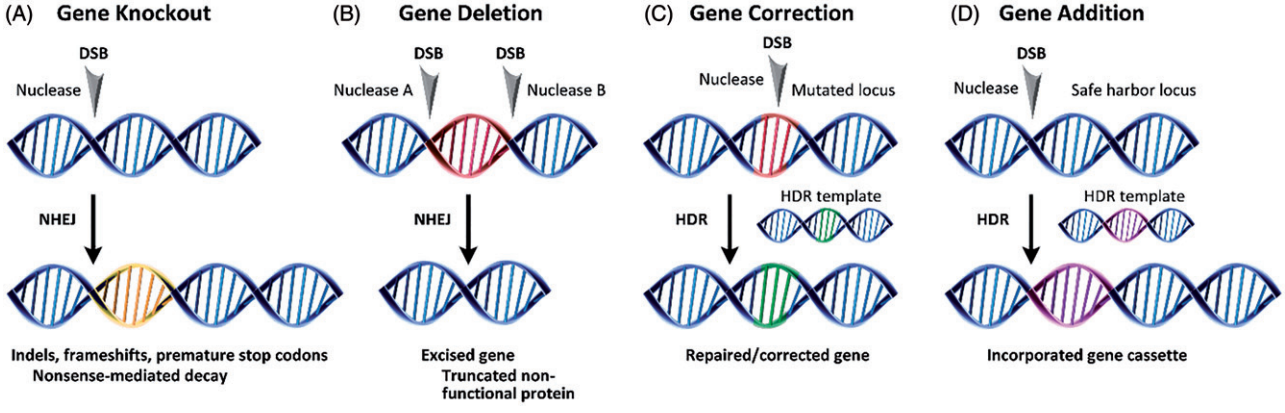

Figure 2. Products of site-specific nuclease-based gene editing: (A) gene knockout, (B) gene deletion, (C) gene correction, and (D) gene addition.

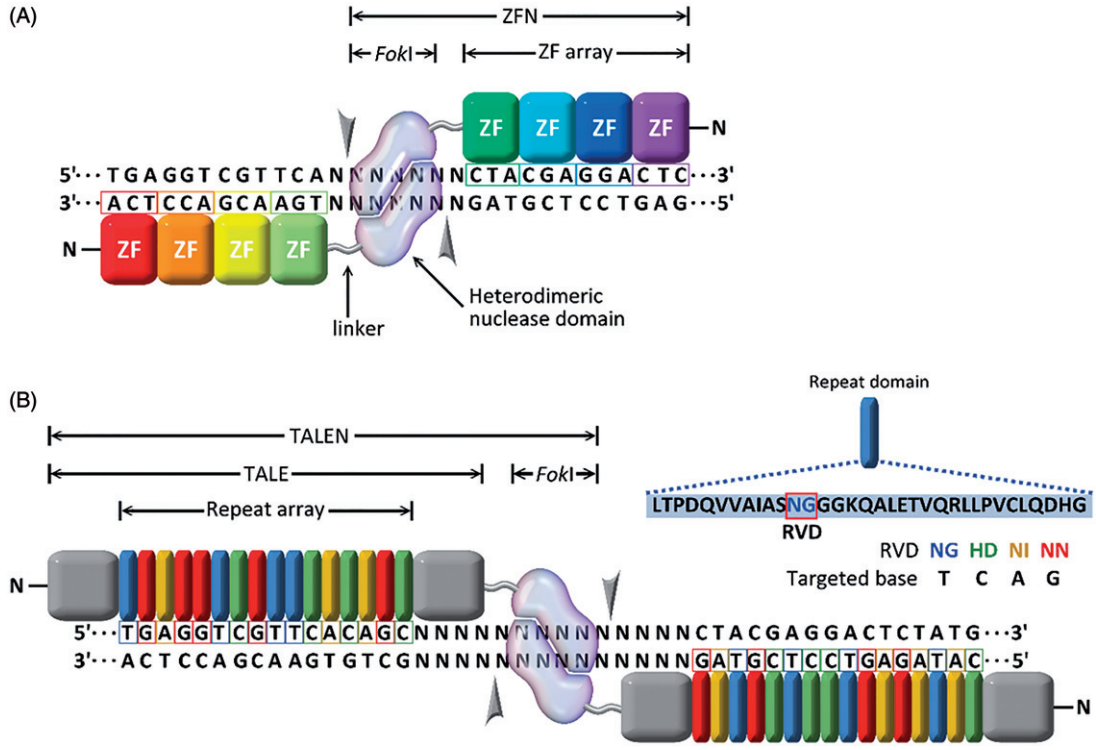

Figure 3. Site-specific endonucleases with programable DNA-binding protein domains: (A) zinc finger nucleases (ZFNs) and (B) transcription activator-like effector nucleases (TALENs).

due to delivery vehicle cargo size limitations. Further, packaging and delivery of TALENs in some viral vectors may be problematic due to the high level of repetition in the TALENs sequence.

CRISPR/Cas9

The most recently developed site-specific gene editing tool, CRISPR/Cas9, is a naturally occurring RNA-guided endonuclease. A methodical investigation by the scientific community has deciphered the natural function of the CRISPR/Cas9 gene editing system. Based on this work, several laboratories developed CRISPR/Cas9 as a tool that has now been applied in much of modern molecular biology. A key difference of this system from the protein-based binding to DNA of ZFNs and TALENs is the use of a short RNA sequence as the specificity-determining element to drive the formation of a DSB at the targeted site. The use of CRISPR/Cas9 avoids the need for protein engineering to develop a site-specific nuclease against a specific DNA target sequence, requiring only the synthesis of a new piece of RNA. This dramatically simplifies and greatly reduces the time needed for gene editing design and implementation.

History of CRISPR

Discovery of unusual repeat sequences in *E. coli* separated by non-repeating sequences in a nearly palindromic pattern was first reported by Ishino et al. (1987). Described as 'curious sequences', similar sequences were identified in *Haloferax* and *Haloarcula* archaea by Mojica et al. (1993) and in *M. tuberculosis* by van Soolingen et al. (1993). The function of the interrupted repeat sequences was unknown, but they were soon identified in 20 microbial species (Mojica et al., 2000) and later found in more than 40% of bacteria and 90% of archaea (Mojica et al., 2005). In 2002, the acronym CRISPR was proposed to bring uniformity to the description of the sequences.

Two significant advances in the understanding of the CRISPR system were then made in 2002, when Jansen discovered a set of genes adjacent to the CRISPR locus, which was termed CRISPR-associated system, or Cas (Jansen et al., 2002). Analysis of the genes indicated a functional relationship between the CRISPR/Cas genes/loci and involvement in DNA metabolism or gene expression. However, the function remained a mystery. The second significant advance occurred in 2005 when Mojica et al. (2005), Pourcel et al. (2005), and Bolotin et al. (2005) all independently reported that the non-repeating CRISPR spacers contained sequences derived from foreign chromosomal DNA, specifically DNA from bacteriophages. Further, some bacteria that carried a given viral DNA sequence in the CRISPR locus were known to be resistant to infection by that phage, indicating that the CRISPR system may be a type of adaptive immune system in prokaryotes. All three studies hypothesized an adaptive immune system function of CRISPR and were rejected by high-profile journals, eventually being published elsewhere (Lander, 2016). The first experimental evidence of this hypothesis was published by Barrangou et al. (2007).

Biological mechanism of CRISPR

Following discovery of the native CRISPR system function in bacteria, researchers set out to understand the mechanism of

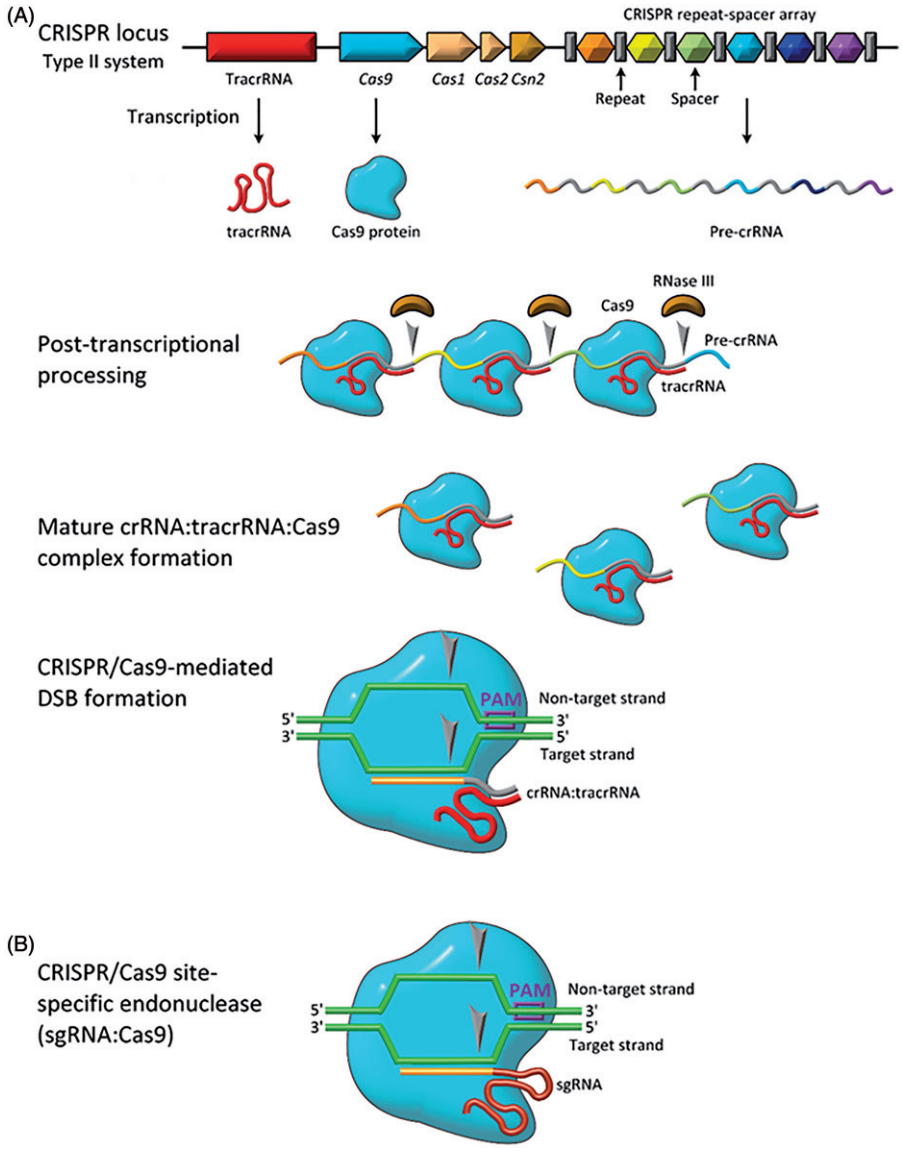

Figure 4. Biology of the type II CRISPR/Cas system. (A) Genomic representation of CRISPR/Cas9 along with relevant transcription/translation products. (B) Engineered CRISPR/Cas9 for site-specific gene editing (sgRNA:Cas9). Grey arrows indicate sites of single-stranded nucleotide breaks.

the adaptive immune system. Although initially hypothesized to follow a RNA interference mechanism (Mojica et al., 2005), it was quickly determined that CRISPR functions as a genomic memory of invading pathogens. This memory is used by Cas proteins, serving as guided endonucleases, to scan for invading DNA and disable it by introducing DSBs (Brouns et al., 2008).

CRISPR systems were further classified into six types that were additionally grouped into two classes (Wiedenheft et al., 2011; Wright et al., 2016). Types I–III are the most studied, while types IV–VI were more recently identified (Makarova & Koonin, 2015; Makarova et al., 2015; Shmakov et al., 2015). Type I and Type III CRISPR systems both utilize sets of Cas proteins. In Type I systems, a multi-protein CRISPR RNA (crRNA) complex known as Cascade recognizes the target DNA, which is then cleaved by Cas3. In Type III systems, Cas10 assembles into a Cascade-like complex that recognizes and cleaves the target.

Type II CRISPR systems require only one protein, Cas9, to scan, bind and cleave the target DNA sequence (Makarova et al., 2011). Details of the Type II CRISPR/Cas9 system are shown in Figure 4(A). The genomic CRISPR locus is comprised of three components: the trans-activating CRISPR RNA (tracrRNA) gene, the Cas gene, and the CRISPR repeat and spacer sequences (Chylinski et al., 2014). These are transcribed into tracrRNA, Cas9 protein, and pre-crRNA. Following transcription, the tracrRNA and pre-crRNA are stabilized by Cas9 and base pair, and RNase III processes the pre-crRNA into crRNA by cleaving it at the repeat (Deltcheva et al., 2011). This dependence on RNase III likely explains why Type II systems are found in bacteria and not archaea, as RNase III is not found in archaea (Garrett et al., 2015). The crRNA:tracrRNA:Cas9 complex forms the active crRNA-guided endonuclease (Chylinski et al., 2013).

The Cas9:RNA complex randomly interrogates DNA in the cell, searching first for the appropriate protospacer adjacent motif (PAM), a short motif (5′-NGG-3′ for Cas9 from

Streptococcus pyogenes) adjacent to the target sequence (Chylinski et al., 2013). Upon recognition of the PAM sequence, the Cas9:RNA complex unwinds the DNA from the first 10–12 nucleotides following the PAM sequence, termed the seed region (Szczelkun et al., 2014). If the interrogated DNA sequence matches the crRNA target sequence, the HNH nuclease domain of the Cas9 nuclease lobe cleaves the target strand while the RuvC-like nuclease domain of the Cas9 α-helical lobe cleaves the non-target strand (Anders et al., 2014; Jinek et al., 2014; Nishimasu et al., 2015). Single mismatches, and sometimes multiple mismatches, can be tolerated by the Cas9:RNA complex, with mismatches being more tolerated in regions downstream of the seed region (Cong et al., 2013; Sternberg et al., 2014).

Type II CRISPR/Cas9 for gene editing

In view of the simplicity of the Type II CRISPR/Cas9 system, researchers first exploited use of CRISPR for gene editing using the Cas9 system from *S. pyogenes*. In 2012, Doudna and Charpentier showed the first use of CRISPR/Cas9 to introduce DSBs in target DNA (Jinek et al., 2012). Further, they showed that the duo-tracrRNA:crRNA units could be engineered into a single, truncated RNA chimera and still direct efficient DNA cleavage. As shown in Figure 4(B), this further simplified CRISPR/Cas9 into a two component system: a Cas9 protein and a single guide RNA (sgRNA). This simplicity makes the CRISPR/Cas9 system the most convenient, simple, and flexible tool for site-directed gene editing currently available.

In early 2013, only a few months after the publication of Jinek et al. (2012), three independent groups reported the use of CRISPR/Cas9 for gene editing. Cho et al. (2013) used the *S. pyogenes* Cas9 system (SpCas9) to edit human cells to incorporate GFP and RFP. Mali et al. (2013) engineered the SpCas9 system and cloned it into human cells, then performed multiplexed editing of target loci. Cong et al. (2013) engineered two different Type II CRISPR/Cas9 systems to introduce precise cleavage in human and mouse cell gene loci. Since these pioneering works, CRISPR/Cas9 has been implemented for gene editing in thousands of laboratories worldwide.

CRISPR: beyond gene editing

In addition to site-specific gene editing, the DNA-binding properties of CRISPR/Cas9 may prove useful in other important applications. Qu et al. repurposed CRISPR into an RNA-guided platform for controlling gene expression by developing a catalytically-dead Cas9 enzyme (dCas9) that retained its capability to recognize and bind a target DNA sequence (Qi et al., 2013). Instead of cleaving the bound DNA, the dCas9 enzyme remained bound to the target DNA sequence, disrupting RNA polymerase or transcription factor binding. They showed that this system, termed CRISPR interference (CRISPRi), could repress expression of multiple genes simultaneously without altering the genome. They demonstrated this gene repression in both *Escherichia coli* and human cells. Shortly after reporting the gene interference CRISPR variant, this same group developed a gene expression/amplification CRISPR (CRISPR activation, CRISPRa) by creating a fusion protein of a dCas9 and a repeating peptide array transcription factor (Tanenbaum et al., 2014). Using these two tools together, genomic libraries of CRISPR inhibition and activation systems were created and used to screen sensitivity to a cholera-diphtheria toxin (Gilbert et al., 2014). Further, Mali et al. (2013) showed that adding functional RNA components to the sgRNA can also enhance transcriptional activity. Two copies of a MS2 RNA aptamer stem-loop sequence were added to the 3′ end of the sgRNA and used together with a Cas9–VP64 activation domain fusion, resulting in robust sequence-specific transcriptional activation. Finally, a system utilizing another catalytically dead Cas9 has been recently described by Gaudelli et al. (2017) for precise base editing. Using a seventh-generation evolved transfer RNA adenosine deaminase attached to dCas9, researchers demonstrated conversion of A*T base pairs to G*C base pairs in a targeted fashion at efficiencies of around 50% with few undesired mutations in human cells. As is the case with CRISPRi and CRISPRa, the Cas9 component is utilized for its precision targeting rather than for catalytic activity. Unlike with those activation and suppression systems, however, the result of this system is gene editing with no double-stranded breaks. As many diseases are caused by single point mutations, this application of CRISPR could prove to be one of the system's most powerful gene editing tools.

The CRISPR system may also serve as a powerful tool for epigenetic studies, allowing for targeted manipulation of epigenetic markers to interrogate epigenetic and transcriptional control relationships. A fusion protein of dCas9 and acetyltransferase was developed by Hilton et al. (2015), catalyzing acetylation of histone H3 lysine 27 at target sites. They showed highly specific gene activation across the genome. Other epigenetic markers (e.g. methyl groups) may be modulated using this approach.

Inducible CRISPR systems were also developed. A photoactivated Cas9 was generated from a split of Cas9 fragments and photoinducible dimerization domains. In response to blue light, the CRISPR/Cas9 system performed gene sequence modification (Nihongaki et al., 2015). Editing activity was extinguished by removing the light source. A similar blue light activating system was developed for the epigenetic gene activator CRISPR system of Hilton et al. discussed above (Polstein & Gersbach, 2015). Chemically induced CRISPRs have also been created. Dow et al. developed a doxycycline-regulated Cas9 that allowed for inducible *in vivo* genome editing in adult mice (Dow et al., 2015).

The specific DNA binding function of CRISPR has also been repurposed to detect the location of genes within undisturbed nuclei of fixed cells (Deng et al., 2015) and living human cells (Ma et al., 2015). Termed CASFISH, dCas9 proteins were labeled with differently colored fluorophores and coordinated to a specific sgRNA. This allowed for multicolor detection of specific genomic loci with high spatial resolution and the assessment of DNA compaction. Unlike traditional fluorescent *in situ* hybridization, this technique avoided the need of heat treatment and chemicals that can distort the

natural organization of the nucleus. It is important to note that fusing proteins to dCas9, however, does not always result in a functional fusion (Ledford, 2016).

Finally, though the CRISPR/Cas9 system has traditionally only been utilized to modify or otherwise interact with DNA substrates containing a PAM site, some recent cutting-edge work suggests that RNA with no PAM site can also be an active substrate for Cas9. Strutt and colleagues demonstrated that Cas9 subtypes II-A and II-C can recognize and cleave RNA in a directed manner utilizing RNA–RNA interactions independent of the presence of a PAM site in the target RNA molecule (Strutt et al., 2018). This cleavage protected *E. coli* cells from infection with bacteriophage MS2 particles, suggesting that Cas9 can provide cellular defense against both DNA and RNA viruses. This exciting work allows for the possibility of direct RNA targeting via CRISPR/Cas9, further expanding the scope of the system for practical applications.

Factors affecting efficacy of the CRISPR/Cas9 system

While the CRISPR/Cas9 system has demonstrated great promise for site-specific gene editing and other applications, there are several factors that influence its efficacy which must be addressed, especially if it is to be used for *in vivo* human gene therapy. These factors include target DNA site selection, sgRNA design, off-target cutting, incidence/efficiency of HDR vs. NHEJ, Cas9 activity, and the method of delivery. As delivery remains the major obstacle for use of CRISPR for *in vivo* applications, efforts addressing other factors will be briefly summarized here. A comprehensive synopsis of existing delivery strategies and potential future delivery candidates will follow.

Target DNA site selection and sgRNA design

A powerful advantage of the CRISPR/Cas9 system is the ability to target any ~23-bp sequence that contains a PAM motif on either strand of DNA. This motif has been reported to occur every eight bps, on an average, for the SpCas9 PAM (Ramakrishna et al., 2014). Cas9 proteins from other species are being characterized and found to have differing PAM sequences. As an example, the PAM from *Neisseria meningitidis* Cas9 is reported to be 5′-NNNNGATT-3′ (Jiang et al., 2013; Ma et al., 2014). This provides even greater flexibility in target sequence selection, and this flexibility will increase as new Cas9 proteins with differing PAMs are identified. Additionally, directed evolution and structure-guided rational design has allowed for engineering of Cas9 variants with altered PAM sequence specificity (Kleinstiver et al., 2015; Anders et al., 2016; Hirano et al., 2016). As examples, the VGR, EQR, and VRER variants of SpCas9 have PAM sequences of 5′-NGAN-3′, 5′-NGAG-3′, and 5′-NGCG-3′, respectively, further reducing limits on genome target selection imposed by the PAM sequence.

Reports from several groups, however, have shown that target site selection and sgRNA design are not as simple as perhaps originally assumed. As mentioned previously, single- and multiple-base mismatches can be tolerated, with mismatches more tolerated at greater distances from the PAM (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013; Wang et al., 2014; Doench et al., 2014; Moreno-Mateos et al., 2015; Xu et al., 2015). One report suggests that CRISPR/Cas9 may be less specific than ZFNs or TALENs due to the relatively shorter targeting sequence (Cradick et al., 2013). This contrasts with many reports that show no detectable off-target cleavage from CRISPR/Cas9 editing, with off-target effects being guide-RNA-specific (Cradick et al., 2013; Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013;Cho et al., 2014). Rational design of the sgRNA has therefore been the subject of a significant body of work resulting in many criteria and no simple rules. There are now many computational tools and software packages available that facilitate sgRNA design. However, caution is still needed, as shown in a recent study by Haeussler et al. (2016) that compared predictions from several sgRNA design tools with experimental results published in eight SpCas9 off-target studies. The authors showed evidence of algorithmic overfitting and the importance of using a model trained on data from the same guide RNA expression system.

Off-target cutting

In addition to rational design of sgRNA, efforts to improve specificity and reduce off-target cutting have resulted in the design of mutant Cas9 systems. While Cas9 itself does not cause off-target effects – these exist solely due to the sgRNA – improvements to the Cas9 protein can limit these effects nonetheless. One mutant system disrupts the Cas9 protein so that it introduces only single-stranded DNA nicks. The nickase CRISPR/Cas9 is then used as a pair with one Cas9 binding to the forward DNA sequence and another Cas9 binding to the reverse DNA sequence flanking the target site. Only when binding in this configuration is a DSB formed through cooperative nicks. Off-target cutting results in only a single-stranded nick that is repaired with simple DNA ligases. The use of this system in mammalian cells reduced off-target cutting by three orders of magnitude with little to no reduction in on-target cutting efficacy (Mali et al., 2013; Ran et al., 2013; Cho et al., 2014).

ZFN and TALEN systems served as inspiration for another mutant Cas9 system, a fusion protein of inactive dCas9 and a *Fok*I nuclease dimer. Again, sgRNAs are designed to bind both the forward and reverse sequences flanking the target, and only when bound in this configuration will the *Fok*I nuclease dimers reconstitute into a functional *Fok*I and form a DSB (Guilinger et al., 2014; Tsai et al., 2014). While these approaches increase specificity and reduce off-target cutting, the number of potential target sites is lower due to PAM and other sgRNA design constraints. This system also significantly increased the size of the gene editing tool, providing greater constraints on *in vivo* delivery approaches. Another reported mutant Cas9 systems designed to reduce off-target effects include fusions of Cas9 with ZFPs or TALEs that can target nearly any genomic locus with improved precision (Bolukbasi et al., 2015). Cas9 mutants have also been designed to reduce non-specific DNA contacts by weakening binding of

the target DNA strand (Kleinstiver et al., 2016) or the non-target DNA strand (Slaymaker et al., 2016) to Cas9 while maintaining robust on-target cleavage.

Incidence/efficiency of HDR

The incidence of HDR-mediated DNA repair from DSBs is typically extremely low in mammalian cells. For example, Cas9-based gene editing in mice resulted in HDR repair efficiencies of 0.5–20%, while NHEJ-mediated repair occurred at 20–60% (Maruyama et al., 2015). Even in the presence of donor template DNA, NHEJ is the more frequent repair mechanism observed from CRISPR/Cas9 editing (Maruyama et al., 2015). Several approaches have emerged to increase HDR efficiency and suppress NHEJ, including use of small molecular inhibitors of NHEJ (Srivastava et al., 2012; Tomkinson et al., 2013; Robert et al., 2015; Vartak & Raghavan, 2015; Yu et al., 2015), gene silencing (Chu et al., 2015), cell cycle synchronization (Lin et al., 2014), and use of cell lines deficient in NHEJ components (Weinstock and Jasin, 2006). One of the most commonly used inhibitors, Scr7, targets the NHEJ component DNA ligase IV, and has been reported to increase efficiency of HDR from Cas9 editing by up to 19-fold (Srivastava et al., 2012; Chu et al., 2015; Vartak & Raghavan, 2015). While the use of Scr7 and other inhibitors have resulted in increased HDR-mediated gene editing efficiency, these inhibitors may have toxic effects on the host cells. Recent work to synchronize cells into late S and G_2 phase, where HDR can occur, along with direct nucleofection of Cas9 ribonuclease complex, may prove a viable alternative to chemical suppression of NHEJ (Lin et al., 2014).

Cas9 activity

Several Cas9 proteins from differing species have been identified and used for gene editing, including *Staphylococcus aureus* (SaCas9) (Ran et al., 2013), *Neisseria meningitidis* (NmCas9) (Hou et al., 2013), and *S. thermophiles* (St1Cas9) (Kleinstiver et al., 2015). Each has differing PAM sequences and variable activity. Thus, selection of a specific Cas9 ortholog may provide improved gene editing efficiency for a given target sequence and should be considered as part of gene editing system design.

In addition to the inherent activity of a given Cas9 protein, other factors have been shown to influence activity. For gene editing in eukaryotic cells, Cas9 must translocate into the nucleus. In these systems, the nuclear location signal (NLS) is connected to the Cas9 protein. Increasing access to the NLS by adding a 32 amino acid spacer between the NLS and Cas9 was shown to increase DNA cleavage activity (Shen et al., 2013). Increasing the relative concentration of sgRNA to Cas9 protein was also shown in increase on-target cutting activity, presumably by ensuring all Cas9 proteins formed the active ribonucleoprotein complex (Kim et al., 2014). However, excessive sgRNA was also shown to increase off-target effects (Fu et al., 2013).

Finally, in comparison with other enzymes, the activity of Cas9 is quite low, with a single turnover rate of $\sim$0.3–1.0 min^{-1}(Jinek et al., 2012). And, once bound to the target DNA sequence, displacement of Cas9 from the DNA strand, even after DSB formation, is challenging – 1 nM Cas9 cleaved $\sim$2.5 nM plasmid DNA after 120 min (Jinek et al., 2012). Thus, Cas9 is less like a catalytic enzyme and more like a single-shot actuator. While this characteristic may be useful in some instances, such as gene activation/inhibition or short-lived activity for gene editing with lower off-target effects, it may be undesirable for other applications where catalytic activity is useful.

CRISPR delivery systems

Here we will discuss the features of the most widely used systems for delivery of CRISPR/Cas9 components. Delivery can be broken into two major categories: cargo and delivery vehicle. Regarding CRISPR/Cas9 cargoes, there are three approaches that are commonly reported: (1) DNA plasmid encoding both the Cas9 protein and the guide RNA, (2) mRNA for Cas9 translation alongside a separate guide RNA, and (3) Cas9 protein with guide RNA (ribonucleoprotein complex). The delivery vehicle used will often dictate which of these three cargos can be packaged, and whether the system is usable *in vitro* and/or *in vivo*. As an example, Cas9 protein is positively-charged, but oligonucleotides and Cas9:sgRNA RNP are negatively charged (Sun et al., 2015). Additionally, considerations for how tightly controlled the overall concentration of Cas9 is must also be made; by introducing Cas9 DNA instead of protein, it becomes more difficult to ascertain precisely how many functional Cas9 units are present in the system at any given timepoint.

Vehicles used to deliver the gene editing system cargo (Table 1) can be classified into three general groups: physical delivery, viral vectors, and non-viral vectors. The most common physical delivery methods are microinjection and electroporation, while methods such as hydrodynamic delivery are currently under investigation. Viral delivery vectors include specifically engineered adeno-associated virus (AAV), and full-sized adenovirus and lentivirus vehicles. Especially for *in vivo* work, viral vectors have found favor and are the most common CRISPR/Cas9 delivery vectors. Non-viral vector delivery is not as prominent as viral-based delivery; however, non-viral vectors possess several advantages over viral vectors and are a bourgeoning area of research. Non-viral vector systems include systems such as lipid nanoparticles, cell-penetrating peptides (CPPs), DNA 'nanoclews', and gold nanoparticles. There are additionally many delivery technologies that have not been demonstrated in the literature as suitable to CRISPR/Cas9 delivery, though they appear to naturally lend themselves to the application. Four such technologies are streptolysin O, multifunctional envelope-type nanodevices (MENDs), lipid-coated mesoporous silica particles, and other inorganic nanoparticles.

Physical delivery methods

Microinjection

Microinjection is considered the 'gold standard' for introducing CRISPR components into cells, with efficiencies

Table 1. CRISPR delivery vehicles and their common features. Relatively difficulty is a subjective measure of how difficult the delivery vehicle is to utilize overall on a four-point scale, where one point is 'few reagents, facile kit provided' and four points is 'requires expert in field with significant experimental experience'.

Delivery vehicle	Composition	Most common cargo	Capacity	Advantages	Limitations	Ease of use	Text refs
Microinjection	Needle	DNA plasmid; mRNA (Cas9 + sgRNA); Protein (RNP)	nM levels of Cas9 and sgRNA	Guaranteed delivery into cell of interest	Time-consuming; difficult; generally *in vitro* only	****	Yang et al. (2013), Horii et al. (2014), Chuang et al. (2017), Nakagawa et al. (2015), Crispo et al. (2015), Raveux et al. (2017), Sato et al. (2015), Ma et al. (2014), Niu et al. (2014), Wu et al. (2013), Long et al. (2014), Ross (1995)
Electroporation; nucleofection	Electric current	DNA plasmid; mRNA (Cas9 + sgRNA)	nM levels of Cas9 and sgRNA	Delivery to cell population; well-known technique	Generally *in vitro* only; some cells not amenable	*	Hashimoto & Takemoto (2015), Chen et al. (2016), Qin et al. (2015), Matano et al. (2015), Paquet et al. (2016), Ousterout et al. (2015), Schumann et al. (2015), Wu et al. (2015), Ye et al. (2014), Choi et al. (2014), Wang et al. (2014), Zuckermann et al. (2015), Kim et al. (2014)
Hydrodynamic delivery	High-pressure injection	DNA plasmid; Protein (RNP)	nM levels of Cas9 and sgRNA	Virus-free; low cost; ease	Non-specific; traumatic to tissues	**	Yin et al. (2014), Guan et al. (2016), Xue et al. (2014), Lin et al. (2014), Zhen et al. (2015), Dong et al. (2015)
Adeno-associated virus (AAV)	Non-enveloped, ssDNA	DNA plasmid	<5kb nucleic acid	Minimal immunogenicity	Low capacity	***	Yang et al. (2013), Long et al. (2016), Carroll et al. (2016), Platt et al. (2014), Hung et al. (2016), Swiech et al. (2015), Chew et al. (2016), Truong et al. (2015), Ran et al. (2015), Nelson et al. (2016), Tabebordbar et al. (2016), Esvelt et al. (2013)
Adenovirus	Non-enveloped, dsDNA	DNA plasmid	8kb nucleic acid	High efficiency delivery	Inflammatory response; difficult scaled production	***	Voets et al. (2017), Maddalo et al. (2014), Wang et al. (2015), Ding et al. (2014), Maggio et al. (2016), Li et al. (2015), Cheng et al. (2014)
Lentivirus	Enveloped, RNA	DNA plasmid	~10kb, up to 18 kb nucleic acid	Persistent gene transfer	Prone to gene rearrangement; transgene silencing	***	Shalem et al. (2014), Wang et al. (2014), Naldini et al. (1996), Kabadi et al. (2014), Heckl et al. (2014); Roehm et al. (2016), Koike-Yusa et al. (2014), Ma et al. (2015), Zhang et al. (2016), Platt et al. (2014)
Lipid nanoparticles/ liposomes/ lipoplexes	Natural or synthetic lipids or polymers	mRNA (Cas9 + sgRNA); Protein (RNP)	nM levels of Cas9 and sgRNA	Virus-free; simple manipulation; low cost	Endosomal degradation of cargo; specific cell tropism	**	Yin et al. (2016), Wang et al. (2016), Zuris et al. (2015), Horii et al. (2013), Sakuma et al. (2014), Schwank et al. (2013), Liu et al. (2014), Liang et al. (2015), Kennedy et al. (2014), Miller et al. (2017), Ebina et al. (2013)
Cell-penetrating peptides (CPPs)	Short amino acid sequences	Protein (RNP)	nM levels of Cas9 and sgRNA	Virus-free; can deliver intact RNP	Variable penetrating efficiency	**	Ramakrishna et al. (2014), Axford et al. (2017)
DNA nanoclew	DNA spheroid	Protein (RNP)	nM levels of Cas9 and sgRNA	Virus-free	Modifications for template DNA needed	****	Sun et al. (2015), Sun et al. (2014)
Gold nanoparticles (AuNPs)	Cationic arginine-coated AuNP	Protein (RNP)	nM levels of Cas9 and sgRNA	Inert; membrane-fusion-like delivery	Nonspecific inflammatory response	**	Mout et al. (2017), Lee et al. (2017)
iTOP	Hyperosmlality + transduction compound	Protein (RNP)	nM levels of Cas9 and sgRNA	Virus-free; high-efficiency	Non-specific; no *in vivo* use yet reported	***	D'Astolfo et al. (2015)
SLO	Bacterial pore-forming toxin	~100kDa proteins and complexes	Unknown for CRISPR	Reversible pore formation; no impact on cell viability	Not yet proven with CRISPR	***	Sierig et al. (2003), Walev et al. (2001), Brito et al. (2008), Teng et al. (2017)
MENDs	Poly-lysine core, lipid coating, CPP decoration	Nucleic acids	Unknown for CRISPR	Customizable; readily modified for precise delivery	Not yet proven with CRISPR	****	Kogure et al. (2004), Nakamura et al. (2012)
Lipid-coated mesoporous silica NPs	Mesoporous Si coated with lipid	Small molecules and short RNA sequences	Unknown for CRISPR	Inert; easy modification with targeting moieties	Not yet proven with CRISPR	***	Liu et al. (2009), Du et al. (2014), Durfee et al. (2016), Gonzalez Porras et al. (2016), Mackowiak et al. (2013), Su et al. (2017), Wang et al. (2013)
Inorganic NPs	NPs of various compositions (carbon, silica)	Large proteins, nucleic acids	Unknown for CRISPR	Inert; used for similar applications	Not yet proven with CRISPR	**	Bates & Kostarelos (2013), Luo et al. (2014), Luo & Saltzman (2000)

approaching 100% (Yang et al., 2013; Horii et al., 2014). In this method, either plasmid DNA encoding both the Cas9 protein and the sgRNA, mRNA encoding Cas9 and sgRNA, or Cas9 protein with sgRNA, can be directly injected into individual cells. Using a microscope and a 0.5–5.0 μm diameter needle, a cell membrane is pierced and cargoes are delivered directly to a target site within the cell. This process circumnavigates barriers associated with delivery through extracellular matrices, cell membranes, and cytoplasmic components. Further, microinjection is not limited by the molecular weight of the cargo, which is a significant limiting factor with viral vector delivery systems. This method also allows for the controlled delivery of known quantities of the cargo, improving control over off-target effects. Naturally, microinjection is best suited for *in vitro* and *ex vivo* work only, as the use of a microscope to target individual cells (and precisely inject cargoes to specific locations within them) precludes the use of microinjection in a true *in vivo* setting.

Nucleic acids are by far the most common cargo for microinjection delivery. There are three primary methods for injection of these components: (1) as DNA directly delivered to the cell nucleus, (2) as *in vitro*-transcribed mRNA molecules delivered to the nucleus, or (3) as *in vitro*-transcribed mRNA molecules delivered to the cytoplasm. These different methods have benefits and drawbacks. By placing the DNA encoding both Cas9 and the sgRNA into the nucleus, the cell is free to transcribe and translate the components. This method is preferred by some groups, such as Chuang et al. (2017) and Nakagawa et al. (2015), due to the ability to omit lengthy *in vitro* transcription reactions from the overall process. However, single-stranded DNA is prone to random integration into the host genome, which may disrupt genes, result in constitutive expression of Cas9, and lead to greater off-target effects. Even circularized plasmid DNA can undergo this phenomenon (Yang et al., 2013).

When delivering mRNA, the ideal case is to deliver the sgRNA directly to the nucleus and the Cas9-encoding mRNA to the cytoplasm, facilitating translation and shuttling of Cas9 to the nucleus. Unfortunately, microinjection is a technically challenging and laborious process, making two different microinjections into a single cell impractical. Further, two microinjections, even when separated by several hours, typically results in non-viable cells (Yang et al., 2013). Therefore, microinjections of CRISPR mRNA components often occurs directly into the cytoplasm of the cell; for some examples see Crispo et al. (2015), Raveux et al. (2017), and Sato et al. (2015). This method has the advantage of putting the Cas9 mRNA directly into the cytoplasm, where it can be translated by the cell. sgRNA in the cytoplasm is then bound by Cas9 while being shuttled into the nucleus, allowing for modification of the host DNA. The vast majority of studies using microinjection to deliver CRISPR use this approach, including simultaneous knock-out of four genes from a single injection into rat zygotes (Ma et al., 2014), disruption of two genes in cynomolgus monkeys from a single injection into one-cell-stage embryos (see Figure 5(A)) (Niu et al., 2014), correction of a cataract-causing mutation in mice (Wu et al., 2013), and correction of a Duchenne muscular dystrophy (DMD)-causing mutation in mice (Long et al., 2014). With some exceptions, microinjection of CRISPR/Cas9 RNA components into cells results in a finite duration of action of the system, owing to the natural decay of mRNA within eukaryotic cells (Ross, 1995). This is often desirable as it reduces off-target effects.

Microinjection is also the most commonly used method for generating animal models. Injection of the gene editing cargo into zygotes allows for efficient germline modification. In addition, there is evidence that injection of Cas9 mRNA and sgRNA into the zygote cytoplasm is the most efficient method for yielding normal embryos and full-term mouse pups harboring the desired modification (Horii et al., 2014). Microinjection can also be useful for CRISPRa and CRISPRi to provide transient up- or down-regulation of a specific gene within the genome of a mature cell. Microinjection is a well-established technology and its use is widespread, as evidenced by the ability to custom-order microinjected mouse zygotes from facilities such as the Genome Modification Facility at Harvard University (https://gmf.fas.harvard.edu/talen-or-crispr-microinjection).

Electroporation

One of the long-standing physical methods for delivery of gene editing tools into a population of cells is electroporation. This technique utilizes pulsed high-voltage electrical currents to transiently open nanometer-sized pores within the cellular membrane of cells suspended in buffer, allowing for components with hydrodynamic diameters of tens of nanometers to flow into the cell. Electroporation is less dependent on cell type than other delivery techniques and can efficiently transfer cargo into cells that are traditionally difficult to manipulate. Electroporation is most commonly used in an *in vitro* setting, though as with microinjection, *ex vivo* applications are also valid. Owing to the oftentimes-large amounts of voltage needed to be applied across cell membranes, however, electroporation is typically not suitable for *in vivo* applications.

There are many published methods for electroporation of mammalian cells. While these protocols can provide a starting point, mammalian cells are often quite sensitive to precise voltages and current application times. This contrasts with bacterial cells, which are often more tolerant of electroporation. This problem becomes even more prominent when studying zygotes rather than immortalized cell lines.

Several groups have developed technological solutions to increase the prominence of electroporation within the CRISPR/Cas9 community. For example, Hashimoto and Takemoto (2015) built a custom electroporation chamber for 40–50 zygotes which allowed them to achieve both very high levels of CRISPR/Cas9 entry into cells and viable embryos. Other groups have used more standard electroporation cuvettes and methods to deliver CRISPR/Cas9 components with high efficiency to zygotes (Figure 5(B)) (Qin et al., 2015; Chen et al., 2016). In other examples, electroporation was used to deliver plasmid DNA encoding both Cas9 and mRNA to generate colorectal cancer models from Cas9-engineered human intestinal organoids (Matano et al., 2015), generate an early-onset Alzheimer's disease model in human

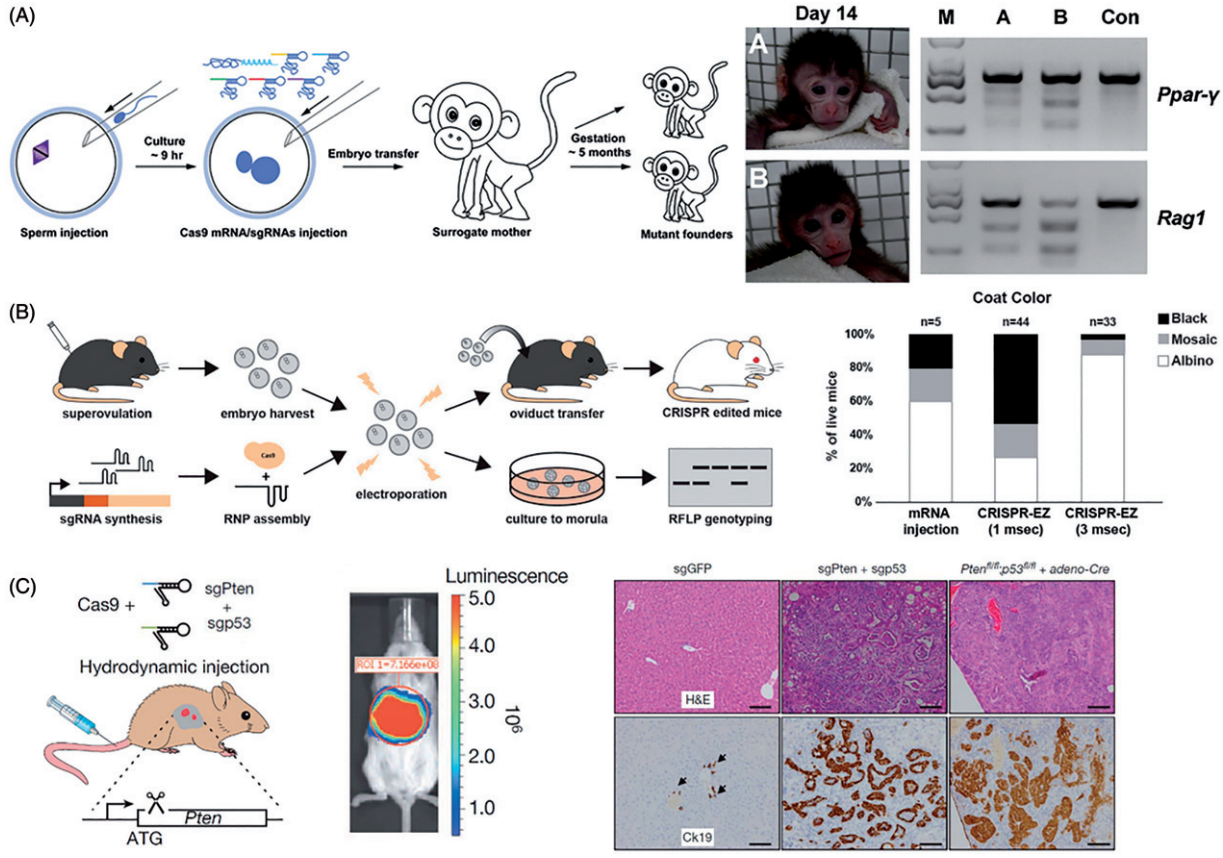

Figure 5. Physical methods for delivery of CRISPR. (A) Microinjection disrupting two genes (*Ppar-γ* and *Rag1*) in Cynomolgus monkeys from a single injection into one-cell-stage embryos. Photographs of Founder Monkeys A and B, PCR products of the targeted loci from genomic DNA of A and B, and a control wild-type Cynomolgus monkey (Con). Adapted with permission from Nui et al. (2014). Copyright 2014 Elsevier Inc. (B) Electroporation delivery of CRISPR RNP targeting genes impacting mice coat color (*Tyr*) followed by transfer to pseudopregnant mothers. Bar plot quantifies coat color phenotypes generated from microinjection and electroporation at 1 ms pulse length and 3 ms pulse length. Adapted with permission from Chen et al. (2016). Copyright 2016 American Society for Biochemistry and Molecular Biology. (C) Hydrodynamic injection of CRISPR into mice results in liver-specific targeting (see bioluminescence image of hydrodynamically injected luciferase plasmid), generating indel mutation of two tumor suppressor genes and oncogenes. The development of liver tumors can be seen in the hematoxylin and eosin (H&E) and cytokeratin 19 (Ck19)-stained micrographs. Adapted with permission from Xue et al. (2014). Copyright 2014 Macmillan Publishers Ltd: Nature.

cells (Paquet et al., 2016), and correct mutations that cause DMD (Ousterout et al., 2015). Electroporation for delivery of Cas9:sgRNA ribonucleoprotein (RNP) to primary human cells has also been reported (Kim et al., 2014; Schumann et al., 2015). The use of RNP was shown to reduce off-target effects versus plasmid transfection and be less stressful on the cells, producing two-fold more embryonic stem cell colonies than with plasmid transfection (Kim et al., 2014).

A specialized electroporation method designed to deliver cargoes directly into the nuclei of mammalian cells has also been used to deliver CRISPR/Cas9. Termed nucleofection, this technique does not require breaking down the nuclear envelope, or cells in a state of division, for cargo to enter the nucleus. Plasmid DNA encoding Cas9 and sgRNA has been delivered via nucleofection to correct a cataract-causing mutation in mouse spermatogonial stem cells (Wu et al., 2015), confer resistance to HIV infection by adding the natural CCR5Δ32 mutation to human cells (Ye et al., 2014), generate lung cancer cell models in human lung epithelial cells (Choi & Meyerson, 2014), and cure latent herpesvirus infection by Cas9-based cleavage and destruction of latent viral genomes (Wang & Quake, 2014).

The use of electroporation and CRISPR/Cas9 to edit genes *in vivo* was also recently reported. Zuckermann et al. (2015) developed a model of a childhood malignant brain cancer, Sonic hedgehog medulloblastoma, via *in utero* electroporation of a developing mouse. Plasmids encoding both Cas9 and sgRNA were injected into the embryo cerebral ventricular zone, followed by electroporation using forceps-like electrodes.

Because of the attractiveness of high-throughput and high-efficiency CRISPR/Cas9 transformation utilizing commonly available laboratory resources, electroporation/nucleofection will likely continue to be used and refined as a major technique to efficiently deliver CRISPR/Cas9.

Hydrodynamic delivery

Hydrodynamic delivery is an *in vivo* delivery technique that involves rapidly pushing a large volume (8–10% body weight) solution containing the gene editing cargo into the bloodstream of an animal, typically using the tail vein in mice. As blood is incompressible, the large bolus of liquid results in an increase in hydrodynamic pressure that temporarily enhances permeability into endothelial and parenchymal cells, allowing for cargo not normally capable of crossing a cellular membrane to pass into cells. This includes naked DNA plasmids and proteins. Delivery of cargo using this

method is significantly enriched in the liver, but also includes cells of the kidneys, lung, muscles, and heart. Hydrodynamic delivery is attractive because it is technically simple and does not require any exogenous delivery components to successfully introduce gene editing components into cells. Hydrodynamic delivery is typically used for *in vivo* applications only, as the premise of delivery relies on transiently increasing the pressure in a closed system and forcing cargo through otherwise-impermeable barriers.

Yin et al. (2014) demonstrated successful delivery of DNA plasmid encoding Cas9 and sgRNA to liver cells using hydrodynamic delivery, resulting in *in vivo* correction of the *Fah* mutation in mouse hepatocytes modeling hereditary tyrosinemia. Although initial delivery efficiency was only one in 250 liver cells, the liver's regenerative capacity allowed for the expansion of the modified cells and phenotype rescue. Soon after, Guan et al. also used hydrodynamic delivery of naked plasmid DNA encoding CRISPR/Cas9 components to a mouse model of hemophilia B. Again, targeting the liver, they showed restored hemostasis in treated mice (Guan et al., 2016). They also showed that an adenovirus (AdV) delivery system resulted in higher corrective efficiency, but no therapeutic effects due to severe hepatic toxicity, presumably a result of the high immunogenicity of the viral vector. Other examples of hydrodynamic injection of plasmid DNA encoding Cas9 and sgRNA include indel mutation of two tumor suppressor genes and oncogenes resulting in generation of liver tumors in mice (Figure 5(C)) (Xue et al., 2014), inhibiting hepatitis B virus (HBV) replication and gene expression in HBV-infected mice (Lin et al., 2014; Zhen et al., 2015), and specific targeting of the HBV genome in the nucleus of HBV-infected mice, showing the potential of CRISPR/Cas9 as a therapeutic against chronic HBV infection (Dong et al., 2015).

Despite these successes, hydrodynamic delivery is not currently being considered for clinical applications. The process of hydrodynamic delivery can be quite traumatic, resulting in potential physiological complications, including cardiac dysfunction, elevated blood pressure, and liver expansion (Suda et al., 2007; Bonamassa et al., 2011). It is relatively easy to cause accidental mortality with this method. Also, as discussed previously, transfection rates are very low, and only certain cell types are amenable to successful delivery.

Viral vector delivery methods

Adeno-associated virus (AAV)

AAV, of the *Dependovirus* genus and *Parvoviridae* family, is a single stranded DNA virus that has been extensively utilized for gene therapy (Daya and Berns, 2008; Samulski and Muzyczka, 2014). AAV is an excellent vehicle for gene therapy for many reasons. AAV is not known to cause or relate with any diseases in humans. There is also a wide range of known serotypes which allow for infection of a multitude of cells with different specificities. The virus itself is able to efficiently infect cells while provoking little to no innate or adaptive immune response or associated toxicity, at least upon first treatment with a serotype (Daya and Berns, 2008). Immune responses are eventually seen to the capsid, sometimes even causing CD-8 T-cell toxicity (Samulski and Muzyczka, 2014). However, owing to the many serotypes of AAV with broad tropism, it is often possible to evade the problem of immune response to AAV should it arise. Finally, unlike some other methods, the use of AAV for gene therapy provides a persistent source of the provided DNA, as AAV-delivered genomic material can exist indefinitely in cells either as exogenous DNA or, with some modification, be directly integrated into the host DNA (Deyle and Russell, 2009). This can, of course, be either advantageous or disadvantageous depending on the desired goals of a specified modification. AAV particles can see application in *in vitro*, *ex vivo*, and *in vivo* work, making them highly versatile delivery vehicles.

CRISPR/Cas9 AAV particles are typically created in HEK 293 T cells. Once particles with specific tropism have been created, they are used to infect the target cell line much in the same way that native viral particles do. This is what ultimately allows for persistent presence of CRISPR/Cas9 components in the infected cell type, and what makes this version of delivery particularly suited to cases where long-term expression is desirable.

With specific regard to CRISPR/Cas9, AAVs are typically utilized as a delivery system in one of the four ways. In the first, SpCas9 and sgRNA are packaged directly onto one DNA plasmid vector and delivered via one AAV particle. While this is within the realm of technical possibility, the SpCas9 and sgRNA are roughly 4.2kB in size, and the overall size of AAV ($\sim$20 nm) only allows for $\sim$4.5–5 kb of genomic material to be packaged within it (Wu et al., 2010). This makes consistent packaging of this construct challenging, and it is also extremely difficult to include other elements (such as reporters, fluorescent tags, multiple sgRNAs, or DNA templates for HDR) to help ensure successful delivery of CRISPR/Cas9 components to cells and meet desired gene editing objectives. This has been accomplished before by Long et al. (2016) who utilized a mini-cytomeglovirus promoter/enhancer with SpCas9 to correct DMD-causing mutations in mice (Figure 6(A)). AAVs were delivered by intraperitoneal, intramuscular, or retro-orbital injection and resulted in muscle function enhancement to varying degrees.

In another approach, AAVs were used to deliver sgRNAs into cells that were previously engineered to express Cas9. Carroll et al. (2016) used microinjection to transfect mouse embryos to express Cas9 in cardiomyocytes and then used AAVs to deliver sgRNAs, resulting in a cardiovascular research model that allows for rapid introduction of indels in heart tissue. Platt et al. developed Cre-dependent Cas9 knock-in mice and used AAVs to deliver sgRNAs, inducing loss-of-function mutations in tumor suppressing genes and gain-of-function mutations in proto-oncogens (see Figure 6(B)). This resulted in the generation of lung adenocarcinoma (Platt et al., 2014).

Many groups have reported success packaging the SpCas9 and sgRNA into two separate AAV particles and using them for co-infection (Swiech et al., 2015; Hung et al., 2016). This has the added benefit of increasing the overall size of the constructs that can be used. However, this naturally adds more complexity than exists with a single vector. Multiple

tags (one for each particle) can be employed to preemptively screen cells for co-infection. In a similar approach, a split Cas9 system has been developed in which the Cas9 C-terminal is packaged into one AAV vector and the Cas9 N-terminal is packaged into a second AAV vector (Figure 6(c)) (Truong et al., 2015; Chew et al., 2016). Reconstitution of the two Cas9 halves results in a functional Cas9 with editing efficiency comparable to the native Cas9, allowing for the use of larger overall Cas9 variants with AAV particles.

The most recently developed AAV CRISPR/Cas9 delivery method was reported by Ran et al. (2015), and it uses a version of Cas9 from *S. aureus* rather than *Streptococcus pyrogenes* (designated SaCas9). This version of Cas9 is roughly 70% the size of SpCas9 while retaining the same potent cutting ability. This results in the ability to use a single vector, but the decrease in size leaves ~1 kB of 'free space' within the AAV particle. This is often enough to include multiple different tags and markers in one particle. Groups have used of SaCas9 in AAV vectors to target the cholesterol regulatory gene Pcsk9 (Ran et al., 2015) and disrupt mutations in the DMD gene in adult mice (Nelson et al., 2016; Tabebordbar et al., 2016). Shorter Cas9 variants from *Streptococcus thermophilus* (Cong et al., 2013) and *Neisseria meningitidis* (Esvelt et al., 2013) have also been used for gene editing and may be good candidates for AAV delivery of CRISPR/Cas9. It should be noted, however, that shorter Cas9 variants identified to date have longer PAM sequences and thus greater limitations on sequences available for targeting.

Lentivirus (LV) and adenovirus (AdV)

While LVs and AdVs are clearly distinct, the way they are utilized for delivery of CRISPR/Cas9 components is quite similar. In the case of LV delivery, the backbone virus is a provirus of HIV (Naldini et al., 1996); for AdV delivery, the backbone virus is one of the many different serotypes of known AdVs. As in the case of AAV, these are plentiful, and finding a useful AdV to a desired target is relatively facile. The serotype most commonly used is AdV type 5. LV is particularly useful because it can be pseudotyped with other viral proteins, such as the G-protein of vesicular stomatitis virus. In doing so, the cellular tropism of the LV can be altered to be as broad or narrow as desired. And, to improve safety, second- and third-generation LV systems split essential genes across three plasmids, significantly reducing the likelihood of accidental reconstitution of viable viral particles within cells. Both LV and AdV can infect dividing and non-dividing cells; however, unlike LV, AdV does not integrate into the genome. This is advantageous in the case of CRISPR/Cas9-based editing for limiting off-target effects. As is the case with AAV particles, both LV and AdV can be used in *in vitro*, *ex vivo*, and *in vivo* applications, which eases both efficacy and safety testing.

In terms of mechanism, this class of CRISPR/Cas9 delivery is like AAV delivery described above. Full viral particles containing the desired Cas9 and sgRNA are created via transformation of HEK 293 T cells. These viral particles are then used to infect the target cell type. The biggest difference between LV/AdV delivery and AAV delivery is the size of the particle; both LVs and AdVs are roughly 80–100 nm in diameter. Compared with the ~20 nm diameter of AAV, larger insertions are better tolerated in these systems. When considering CRISPR/Cas9, additional packaging space for differently-sized Cas9 constructs or several sgRNAs for multiplex genome editing is a significant advantage over the AAV delivery system.

Many groups are currently using AdV or LV vectors for delivery of CRISPR/Cas9 components. Voets et al. (2017) recently used an AdV vector to inactivate genes in normal human lung fibroblasts and bronchial epithelial cells, wherein they reported efficient silencing of genes at MOIs of AdV as low as 20. Additionally, Kabadi et al. (2014) created a unique lentiviral CRISPR/Cas9 system via Golden Gate synthesis. Their construct allowed for the expression of one Cas9 and four different sgRNAs, each under the control of a different promoter, to allow for the editing of several different types of human cells. The work done by these two groups, especially on primary human cells, allows for exciting possibilities for the use of these delivery systems *in vivo*.

Maddalo et al. (2014) reported the generation of a model of *Eml4-Alk* oncogene-driven lung cancer in adult mice by intratracheal instillation of AdV-delivered CRISPR/Cas9. Wang et al. (2015) used AdV delivery of Cas9 and sgRNA to target *Pten*, a gene involved in the liver disease non-alcoholic steatohepatitis (NASH) as shown in Figure 6(D). Four months post treatment, *Pten* gene-edited mice showed massive hepatomegaly and features of NASH. Importantly, in addition to displaying AdV vector-associated immunotoxicity in the liver, humoral immunity against SpCas9 was detected, as was a potential SpCas9-specific cellular immune response. This indicates the importance of also studying the immunogenicity of specific Cas9s for *in vivo* delivery of CRISPR.

The use of AdV for CRISPR/Cas9 delivery has been reported targeting loss-of-function *PCSK9* mutation in mouse liver (Ding et al., 2014), *in vitro* partial restoration of muscle function in a DMD model mice (Maggio et al., 2016), resistance to HIV-1 infection of primary CD4 + T-cells by adding the cell membrane receptor *CCR5*Δ32 variant (Li et al., 2015), and tissue-specific gene knockout in mouse liver (Cheng et al., 2014).

Examples of LV delivery of CRISPR/Cas9 include modification of up to five genes using a single LV to deliver plasmid DNA encoding Cas9, sgRNA and a fluorescent marker to develop a mouse model of acute myeloid leukemia (Heckl et al., 2014), target of herpes simplex virus-1 genome regions essential to viral protein expression during early and late phases of viral infection/reinfection to suppress infection and prevent new infection (Roehm et al., 2016), and the use of LV to mutate genes in mouse primary immune cells (Platt et al., 2014).

Leveraging the integration ability of LV, this system has also been used to create gene libraries for studying mechanisms of disease. Wang et al. (2014) used LV to deliver a pool of 73,000 sgRNAs to two human cells lines that had been previously engineered to express Cas9. Shalem et al. (2014) delivered 64,751 unique sgRNA sequences by LV to human cancer cells, and Koike-Yusa et al. (2014) delivered 87,897 sgRNAs targeting 19,150 protein coding genes in mouse

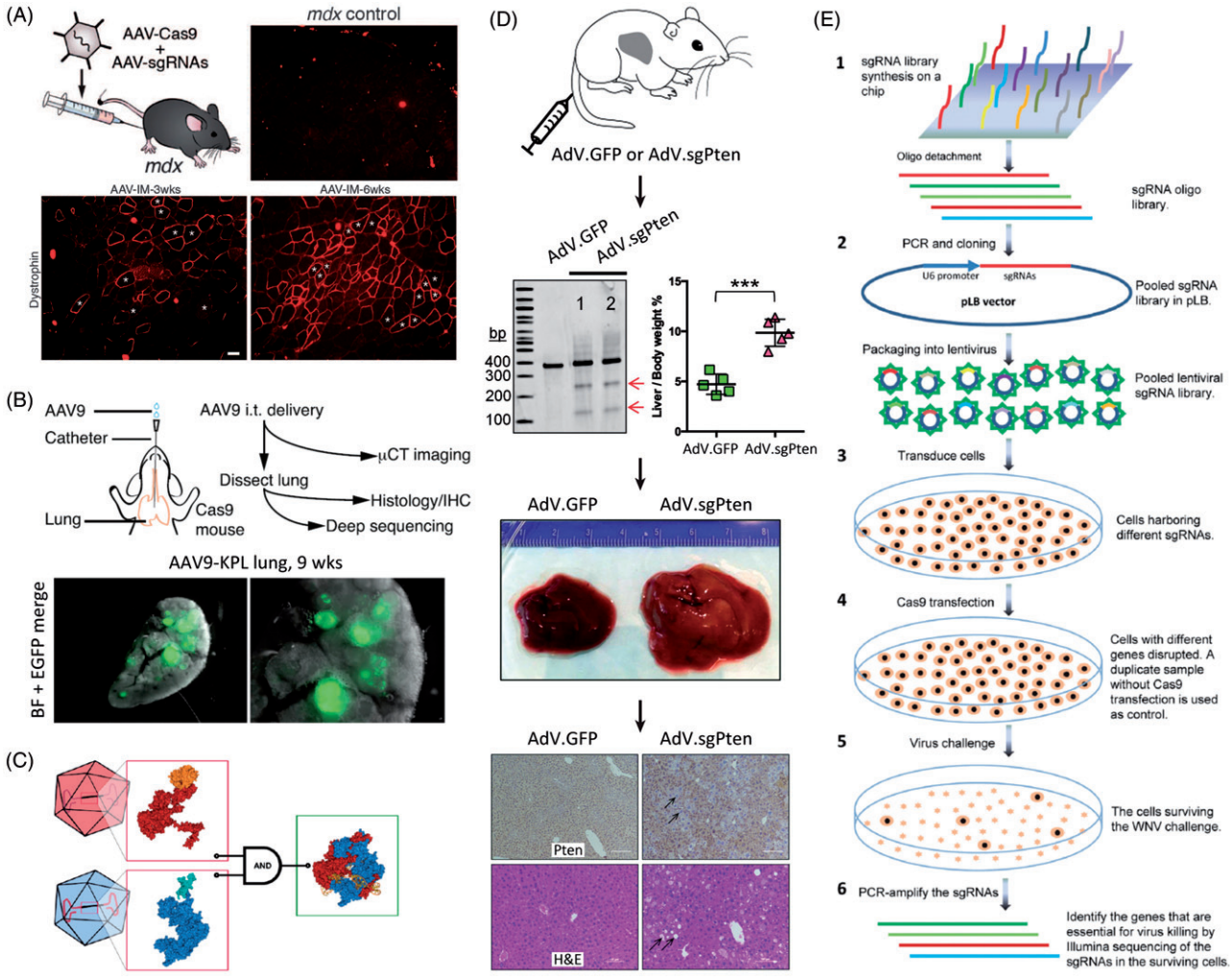

Figure 6. Viral vector methods for delivery of CRISPR. (A) AAV delivery of Cas9 and sgRNAs disrupting mutations in the Dmd gene in adult *mdx* mice, resulting in improvement of muscle biochemistry and function. Adapted with permission from Long et al. (2016). Copyright 2016 American Association for the Advancement of Science. (B) AAV intratracheal instillation delivery of sgRNAs in Cre-dependent Cas9 knock-in mice, resulting in lung adenocarcinoma (EGFP-positive tumors). Adapted with permission from Platt et al. (2016). Copyright 2014 Elsevier Inc. (C) A split Cas9 system in which the Cas9 C-terminal is packaged into one AAV vector and the Cas9 N-terminal is packaged into a second AAV vector. Reconstitution results in a fully functioning Cas9. Reprinted from Truong et al. (2015). Copyright 2014 The Authors (CC BY license). (D) AdV delivery of Cas9 and sgRNA targeting the *Pten* gene in mouse liver resulting in *Pten* mutation (see arrows by gel), and massive hepatomegaly and features of NASH in infected livers. Immunohistochemistry shows loss of Pten staining (arrows) one month after AdV infection; H&E stained micrographs show sections of steatosis (lipid accumulation, arrows) four months post infection. Adapted with permission from Wang et al., 2015. Copyright 2015 Mary Ann Liebert, Inc. Publishers. (E) Schematic of a lentivirus and CRISPR-based gene library functional screen used to identify the genes essential for West-Nile-virus-induced cell death. Reprinted from Ma et al. (2015). Copyright 2015 The Authors (CC BY license).

embryonic stem cells, both again constitutively expressing Cas9. These, and other similar studies, will enable identification of new therapeutic targets and the design of next-generation drugs. For example, genomics screening using CRISPR/Cas9 and LV was used to identify the genes essential for West-Nile-virus-induced cell death (Figure 6(e)) (Ma et al., 2015) and define a signal peptide pathway required by flaviviruses (Zhang et al., 2016).

However, there are drawbacks to LV or AdV delivery systems, as typical AdVs and LVs elicit strong immune responses (Follenzi et al., 2007; Ahi et al., 2011). In addition, although care is taken to make the HIV provirus as integration-deficient as possible (Chen and Goncalves, 2016) and AdVs are naturally very low integrators into the cell genome, it is not currently possible to completely eliminate the chances of integration into the host. Additionally, although steps can be taken to make this integration targeted, one cannot guarantee that the viral payload goes to the same precise location every time. This can result in an increase of expression and off-target effects, or even potential damage to the cell if the insertion randomly occurs within an important cellular protein (Bestor, 2000; Papapetrou and Schambach, 2016). Care must always be taken with LVs and AdVs when utilizing them for genome editing.

Non-viral vector delivery vehicles

Lipid nanoparticles/liposomes

Lipid nanoparticles have long been used as delivery vehicles for a wide range of different molecules to cells and have demonstrated popularity for nucleic acid delivery. Nucleic acids are typically unstable outside of cells, and owing to their highly anionic nature, they do not easily pass through

the cell membrane. However, by encapsulating nucleic acids within typically very cationic liposomes, they can be delivered to cells with relative ease. Lipid nanoparticles do not contain any viral components, which helps minimize safety and immunogenicity concerns. They can also, like viral particles, be utilized *in vitro*, *ex vivo*, and *in vivo*, allowing for extensive testing on a variety of scales of cell populations.

When used to deliver CRISPR/Cas9 components, there are two main approaches to the use of lipid nanoparticles: delivering Cas9 and sgRNA genetic material (either plasmid DNA or mRNA) or delivering Cas9:sgRNA RNP complexes. If delivering Cas9 mRNA and sgRNA, this method is functionally like microinjection in result (Yin et al., 2016). However, several groups have shown good success with the use of Cas9:sgRNA RNP complexes (Zuris et al., 2015; Wang et al., 2016). CRISPR/Cas9 seems to be particularly well-suited to this type of delivery because Cas9 and the sgRNA as a ribonucleoprotein complex are highly anionic. This allows them to be packaged utilizing approaches typically employed for delivering nucleic acids.

There are substantial drawbacks for delivery of CRISPR/Cas9 components via lipid nanoparticle. First, there are both external and internal barriers that must be considered. Once the nanoparticle has passed through the surface of the cell, it is typically encased within an endosome. Encased contents can very rapidly be directed by the cell into the lysosomal pathway, causing the degradation of all lysosome contents. Therefore, the cargo must escape the endosome. Also, if the Cas9:sgRNA complex can escape the endosome, it must also translocate to the nucleus, which can also be a potential point of failure. Because of this, it is rare to see particularly high efficacies when delivery CRISPR/Cas9 components via lipid nanoparticles. While Wang et al. (2016) could achieve ~70% *in vitro* modification efficiency in cells (see Figure 7(A)), that only came after an intense screen to determine the most optimal lipids with which to construct their liposomes for their system. Finally, lipid nanoparticles are like virus particles in that the nature and size of the cargo, along with the target cell type, highly affect transfection efficiency and the types of lipids that are appropriate or useful in the system.

Perhaps the most commonly used lipid nanoparticle system is the commercially available Lipofectamine. Lipofectamine is a cationic liposome formulation that complexes to negatively charged nucleic acids, allowing fusion of the complex with negatively-charged cell membranes and endocytosis. Lipofectamine has been used to deliver Cas9- and sgRNA-encoding plasmid DNA to human pluripotent stem cells to generate a model for Immunodeficiency, Centromeric region instability, Facial anomalies syndrome (ICF) syndrome with 63% transfection efficiency (Horii et al., 2013), transfect human cells with an all-in-one expression cassette with up to seven sgRNAs and a Cas9 nuclease/nickase (Sakuma et al., 2014), correct the cystic fibrosis transmembrane conductor receptor locus in cultured intestinal stem cells of cystic fibrosis patients (Schwank et al., 2013), introduce modular 'AND' gate circuits based on CRISPR/Cas9 that detects bladder cancer cells, inhibits bladder cancer cell growth, induces apoptosis, and decreases cell motility (Liu et al., 2014), and deliver Cas9:sgRNA RNP *in vivo* to modify the hair cells within mouse inner ear (Zuris et al., 2015).

In an intriguing study, Liang et al. (2015) compared the transfection efficacy of three lipofectamine formulations and electroporation against eleven cell lines. They also compared different gene editing cargoes: plasmid DNA, Cas9 mRNA with sgRNA, and Cas9:sgRNA RNP. They showed greater efficiencies for electroporation transfection versus lipofectamine, and lower off-target effects using RNP over plasmid DNA or mRNA cargo.

Because of their lack of viral components, there will always be interest in improving lipid nanoparticles to deliver CRISPR/Cas9 components. This improvement process can come through the screening of better lipid carriers, as above; better decorations on the liposome surface to help target particles to specific cells or tissues, avoid immune system detection, and facilitate endosomal escape; and improved packaging of CRISPR/Cas9 components, increasing the odds of some subset of packaged molecules to be appropriately delivered.

Lipoplexes/polyplexes

Delivery of CRISPR/Cas9 gene editing components has been reported using other nanocomplexes that generally rely on electrostatic interactions. A common approach is the use of the commercially available FuGENE-6 reagent, a non-liposomal solution containing lipids and other proprietary components. Kennedy et al. used FuGENE-6 to deliver Cas9 and sgRNA encoding plasmid DNA, inactivating human papillomavirus E6 or E7 gene in cervical carcinoma cells, resulting in cell-cycle arrest and eventual cell death (Kennedy et al., 2014). The synthesis and development of zwitterionic amino lipids (ZALs) was reported by Miller et al. (2017). ZALs were complexed with Cas9 mRNA and sgRNA, forming nanoparticles with ~15 nm diameter which showed effective transfection in mice, accumulating primarily in the liver, kidney, and lungs. Another common and commercially available technique, calcium phosphate transfection, utilizes Ca^{2+} molecules to induce precipitation of DNA/Ca^{2+} microcomplexes. These complexes strongly bind to the negatively charged cell membrane and induce endocytosis into the cell. Ebina et al. (2013) used calcium phosphate transfection to deliver plasmid DNA encoding Cas9 and sgRNA into latent HIV-1-infected human 293 T cells. The CRISPR construct targeted the provirus genome, blocking expression of viral components and removing internal viral genes from the host cell chromosome.

Other common polymeric vectors for DNA delivery are polyethenimine (PEI) and poly(L-lysine) (PLL). Branched PEI have high charge density, facilitating efficient plasmid DNA packing, and pH-buffering ability which enables escape from endosomes. However, branched PEI is cytotoxic. Therefore, a balance between the desirable properties of branched PEI and the less toxic linear PEI must be struck for effective transfection. Zuckermann et al. (2015) reported the use of PEI to deliver Cas9- and sgRNA-encoding plasmid DNA into mouse brains to generate a malignant brain tumor model

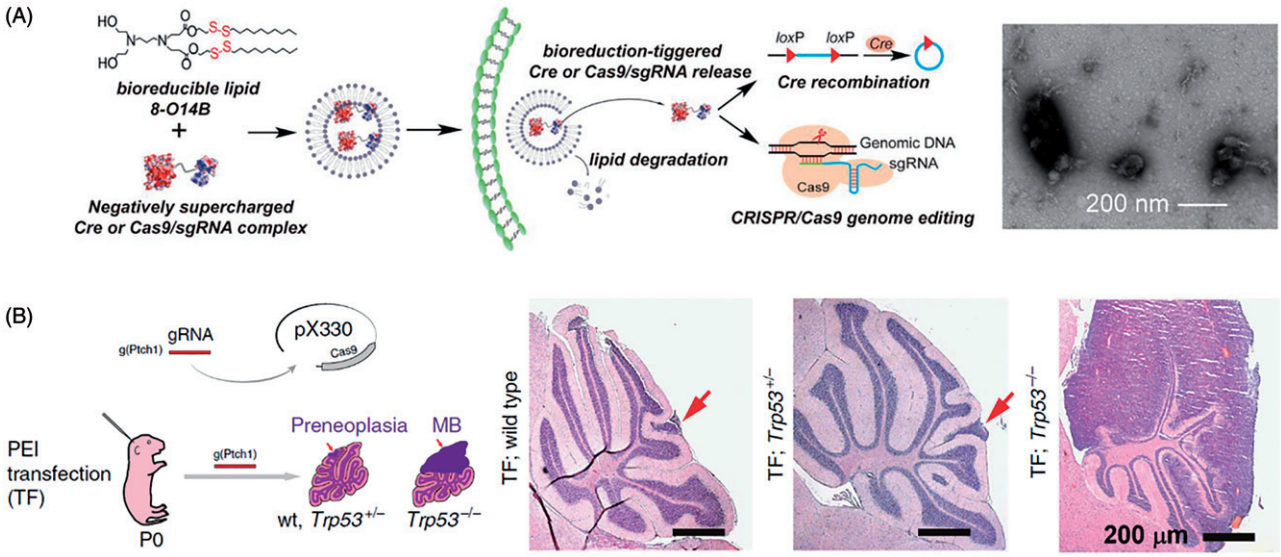

Figure 7. Lipid nanoparticle and polyplex delivery of CRISPR. (A) Combining bioreducible lipid nanoparticles and anionic Cas9:sgRNA complexes drives the electrostatic self-assembly of nanoparticles (see TEM micrograph of 3-O14B/Cas9:sgRNA nanoparticles) for potent protein delivery and genome editing. Adapted with permission from Wang et al. (2016). Copyright 2017 National Academy of Sciences. (B) Microinjection of PEI with Cas9- and sgRNA-encoding plasmid DNA into mouse brain directed against the *Ptch1* locus to generate a malignant brain tumor model. Compare the wild type and *Trp53*± H&E stained micrographs (arrows indicate small lesions encompassing only one cerebellar folium) with the tumor from the $Trp53^{-/-}$ condition (MB = medulloblastoma). Adapted from Zuckermann et al. (2015). Copyright 2015 The Authors (CC BY license).

(Figure 7(B)). PEI was also used to deliver CRISPR plasmid DNA to inhibit HBV replication and gene expression in HBV-infected mice (Zhen et al., 2015). PLL has been used to complex with Cas9 plasmid DNA, forming a multifunctional envelope-type nanodevice (MEND), described in the Emerging Delivery Technologies section of this review.

Cell-penetrating peptides (CPPs)

CPPs are generally short stretches of amino acids that are polycationic, amphipathic, or non-polar in nature. Each class of CPPs can facilitate uptake of different types of proteins into different cell types, and often different combinations of CPPs and the desired molecule for uptake will result in different uptake levels. CPPs can be used for *in vitro* and *ex vivo* work quite readily, and extensive optimization for each cargo and cell type is usually required. Because of the level of detail required for this optimization, CPPs are not generally currently utilized to deliver components *in vivo*. In the specific case of CRISPR/Cas9, the CPPs are usually covalently attached to the Cas9 protein directly, which is then complexed with the sgRNA and delivered to cells. Some work with CPPs and CRISPR/Cas9 was accomplished as early as 2014 by Ramakrishna et al. (2014), who showed separate delivery of CPP–Cas9 and CPP–sgRNA to multiple human cell lines. However, most reports are quite recent, such as the work done by Axford et al. (2017) in which the authors demonstrated cellular and sub-cellular localization of CPP-delivered CRISPR/Cas9 RNP using confocal microscopy.

Typically, CPPs show low efficiency of the desired targeted mutation in cells – usually around 10–20%. As shown above, however, naked plasmids can achieve tangible long-term effects with an efficiency rate of just 0.4%. As CPPs are roughly 40-fold more efficient than transfection from bare plasmids, CPPs are a serviceable method for delivery of CRISPR/Cas9 components to cells. This method requires a fair amount of investment, however, as efficiencies of the CPP themselves to penetrate cellular membranes vary with both attached cargo and cell type. The same challenges of translocating the Cas9:sgRNA complex into the nucleus once it is within the cell must also be overcome.

DNA nanoclew

A DNA 'nanoclew' is a unique technology for CRISPR/Cas9 component delivery. Developed by Sun et al. (2014), a DNA nanoclew is a sphere-like structure of DNA that has been compared with a ball of yarn. The nanoclew is synthesized by rolling circle amplification with palindromic sequences that aide in the self-assembly of the structure. The sphere can then be loaded with a payload – Sun et al. originally used doxorubicin – and the payload can be specifically triggered for release by certain biological conditions. As this is a relatively new delivery technology, it has currently only been utilized in an *in vitro* setting. In 2015, the group repurposed the nanoclew for CRISPR/Cas9 delivery by designing the palindromic sequences to be partially complementary to the sgRNA within the Cas9:sgRNA ribonucleoprotein complex (Figure 8(A-a)) (Sun et al., 2015). By coating the nanoclew with PEI to induce endosomal escape (Figure 8(A-b)), the group demonstrated roughly 36% efficiency in delivery of CRISPR/Cas9 RNP with the nanoclew (compared with 5% with bare Cas9:sgRNA and PEI). This allowed the nanoclew to attain efficiencies on the order of other high-efficiency CRISPR/Cas9 delivery systems, but still contain no viral components (or indeed, any exogenous material besides repeating DNA and PEI). More testing is warranted, particularly on

the potential immunogenicity of DNA nanoclews. Still, early results are promising for this new delivery system.

Gold nanoparticles (AuNPs)

AuNPs have many uses in applied biomedical science, from imaging agents to inert carriers of other components. As such, these particles are readily used in *in vitro*, *ex vivo*, and *in vivo* settings. Mout et al. (2017) demonstrated that, by engineering Cas9:sgRNA RNP and AuNPs to associate with one another (Figure 8(B)), a complex is created that can be efficiently delivered to cells and cause a desired mutation at a rate of roughly 30%. Lee et al. (2017) also reported use of AuNPs to deliver Cas9:sgRNA RNP to mice suffering from DMD. In this work, 15 nm diameter AuNPs were conjugated to thiolated short DNA oligos (Figure 8(C)), which were then conjugated to a single-stranded donor DNA. This donor DNA then complexed with the Cas9 RNP. The resulting particle was coated in a silicate and an endosomal disruptive polymer, PAsp(DET). A single injection of the AuNP-Cas9 conjugate corrected 5.4% of the mutated DMD-causing dystrophin gene and showed recovered dystrophin gene expression. Treated mice further showed partial restoration of muscle function and reduced levels of fibrosis.

Again, these results place AuNPs within the high bounds for CRISPR/Cas9 delivery efficiency while also eliminating the need for exogenous viral material. In addition, unlike the DNA nanoclew which relies on a biological molecule to act as a carrier, AuNPs are inert and will not trigger an immune response to the nanoparticle itself (Lee et al., 2017). Still, AuNPs have been shown to stimulate immune cytokine production in general (for a recent review, see Dykman and Khlebtsov, 2017). While this method also requires additional testing, it is promising as another delivery mechanism for CRISPR/Cas9 components.

iTOP

Many other delivery techniques have been developed for gene editing systems. An example includes the CPP-independent protein delivery method reported by D'Astolfo et al. (2015). This technique, termed iTOP for induced transduction by osmocytosis and propanebetaine, uses NaCl-mediated hyperosmolality together with a transduction compound (propanebetaine) to trigger macropinocytotic uptake into cells of extracellular macromolecules. As another newer delivery technique, applications of iTOP have been limited to *in vitro* settings at this time. iTOP was used to delivery CRISPR/Cas9 RNP into primary human KBM7 and H1 cells, conferring diphtheria toxin resistance with ~70% gene knockout efficiency.

Emerging delivery technologies

We conclude this review with a look at four intriguing technologies: streptolysin O (SLO), multifunctional envelope-type nanodevices (MENDs), lipid-coated mesoporous silica particles, and inorganic nanoparticles. While none of these have been demonstrated in the literature for CRISPR/Cas9 delivery, their properties make them naturally amenable for use as CRISPR/Cas delivery vehicles.

SLO

SLO is a toxin produced by Group A streptococci that works by creating pores in mammalian cell membranes (Sierig et al., 2003). While typically fatal to cells, Walev et al. (2001) developed a system in 2001 to allow for this toxin to act in a reversible manner. This allows for the delivery of proteins of up to 100 kDa to the cytosol of both adherent and non-adherent cells in culture without compromising overall viability. Other groups have used SLO for delivery of siRNA (Brito et al., 2008) and imaging agents for live-cell microscopy (Teng et al., 2017). Although there would be clear challenges to using SLO *in vivo*, the potential is there for its usage *in vitro* for delivery of CRISPR/Cas9 components, primarily for the smaller variants of Cas9.

MENDs

MENDs, developed by the Harashima group at Hokkaido University, are a non-viral gene editing and therapeutic delivery system that is composed of condensed plasmid DNA, a PLL core, and a lipid film shell (Kogure et al., 2004). Packaging the DNA/PLL core with lipids increased transfection rates by ten-fold over bare DNA/PLL. Addition of the cell-penetrating peptide stearyl octaarginine to the lipid shell increased transfection rates by 1000-fold over bare DNA/PLL. The lipid envelope can be readily modified with other functional components, including the following: polyethylene glycol to increase vascular circulation time, ligands for targeting of specific tissues/cells, additional cell-penetrating peptides for greater cellular delivery, lipids to enhance endosomal escape, and nuclear delivery tags.

Recently, a tetra-lamellar MEND (T-MEND) was developed that targeted the cellular nucleus and mitochondria, and a PEG-peptide-DOPE-conjugated MEND (PPD-MEND) was developed that targeted bladder cancer cells (Nakamura et al., 2012). MEND has been used to successfully deliver cargoes of plasmid DNA, short interfering RNA (siRNA), and Bacillus Calmette-Guerin (BCG) cell wall therapeutic agents. This versatile platform may serve as an effective CRISPR/Cas9 delivery tool in the future, although as with SLO, more work must be done to move the work from *in vitro* to *in vivo* settings.

Lipid-coated mesoporous silica particles

Developed by Brinker and colleagues at Sandia National Laboratories and the University of New Mexico, this biological delivery system is composed of a mesoporous silica nanoparticle core and a lipid membrane shell (Liu et al., 2009). While not yet utilized for CRISPR/Cas9, the particles have intriguing properties that may make them good delivery vehicles for the technology. The silica core has a large internal surface area, leading to high cargo loading capacities. In addition, pore size, pore chemistry, and overall

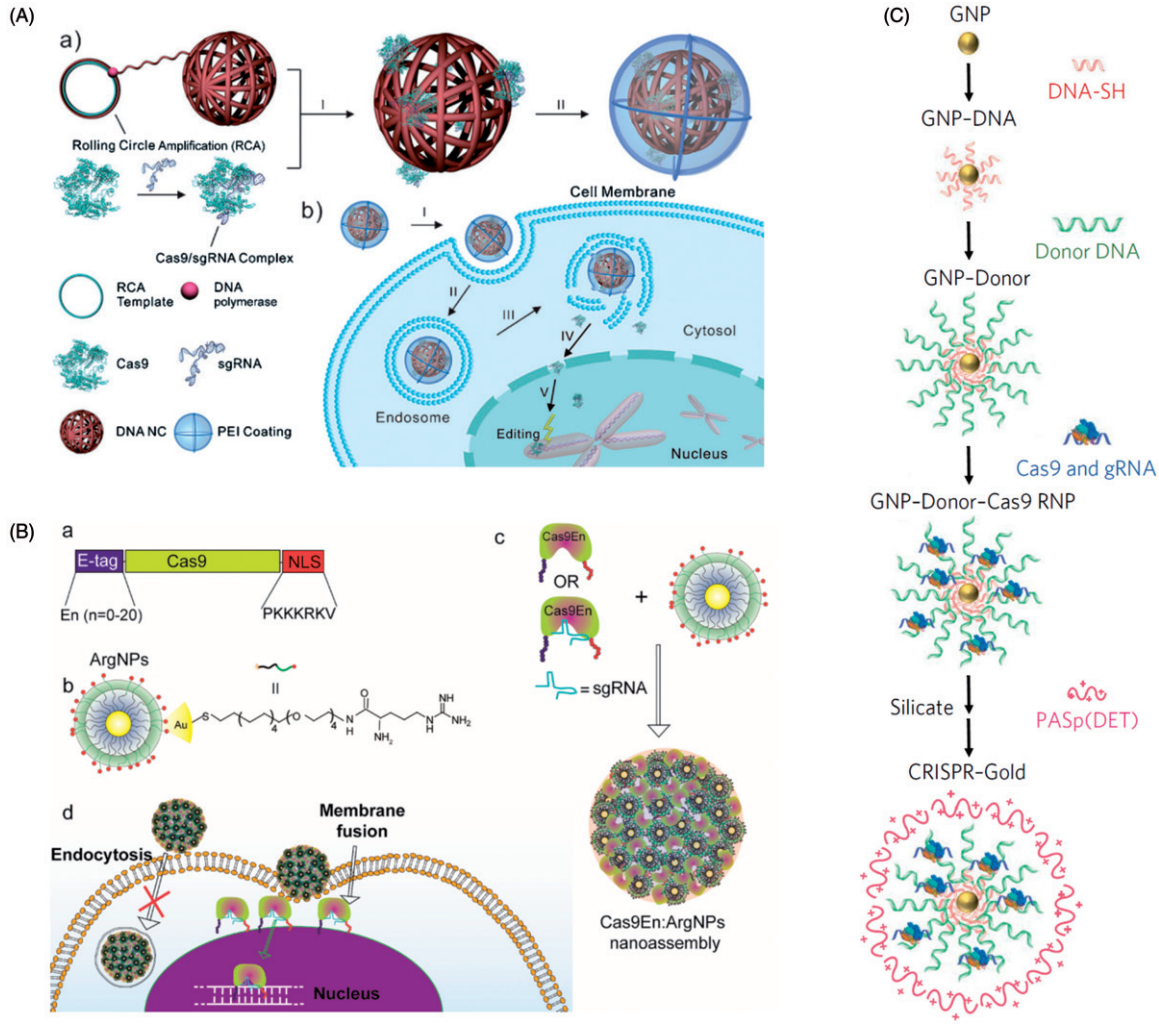

Figure 8. Nanomaterial delivery vehicles for CRISPR delivery. (A) DNA 'nanoclew' loaded with Cas9:sgRNA RNP via Watson-Crick base pairing, followed by coating with PEI to improve endosomal escape. Reprinted with permission from Sun et al. (2015). Copyright 2015 John Wiley & Sons. (B) Arginine-modified gold nanoparticles (ArgNPs, positively charged) interact with multiple Cas9:sgRNA RNPs engineered with an E-tag to form a local negatively charged region, forming a nanoassembly that delivers Cas9 via membrane fusion. Reprinted with permission from Mout et al. (2017). Copyright 2017 American Chemical Society. (C) Synthesis of AuNPs engineered to complex with multiple Cas9:sgRNA RNPs, followed by coating in silica and the endosomal disruptive polymer PASp(DET). Adapted with permission from Lee et al. (2017). Copyright 2017 Macmillan Publishers Ltd: Nature Biomedical Engineering.

particle size can be individually tailored, allowing for the loading of various types of cargo (Du et al., 2014; Durfee et al., 2016). The lipid coating of the particle can also be tailored to maximize cargo loading, increase circulation times, and provide precise targeting and cargo release. A wide variety of lipids and lipid modifications have been used in the formulation of lipid-coated mesoporous silica particles, allowing selection of the most relevant lipid formulation for the selected cargo and application (Liu et al., 2009; Mackowiak et al., 2013; Wang et al., 2013; Du et al., 2014; Durfee et al., 2016; Gonzalez Porras et al., 2016; Su et al., 2017).

To date, lipid-coated mesoporous silica particles have been loaded with a variety of imaging agents, chemotherapeutics, and phototherapy agents for both *in vitro* and *in vivo* work (Mackowiak et al., 2013; Wang et al., 2013; Durfee et al., 2016; Su et al., 2017). The characteristics of this delivery platform seem to naturally lend themselves to CRISPR/Cas9 components. Still, there are many challenges to overcome, primarily the packaging of large cargoes. CRISPR/Cas9 components, whether in an RNP complex, as mRNA, or as DNA plasmids, are larger than other components that have been reported to load within the particles.

Inorganic nanoparticles

Inorganic nanoparticles are natural potential CRISPR component carriers because they have already been used for similar purposes. Examples of these include AuNPs, carbon nanotubes (CNTs), bare mesoporous silica nanoparticles (MSNPs), and dense silica nanoparticles (SiNPs). The use of AuNPs for CRISPR/Cas9 delivery was described above. While CNTs (Bates and Kostarelos, 2013), MSNPs (Luo et al., 2014), and SiNPs (Luo and Saltzman, 2000) have been used for many gene delivery applications, the use of these carries for Cas9 delivery has yet to be reported. However, when compared with viral and lipid/polymer based vectors, inorganic nanoparticles are simpler to generate, with reproducible composition, size, and size distribution, are simpler to characterize and chemically functionalize, and are more stable over time. We, therefore, expect many reports detailing the use of inorganic

nanoparticles as delivery vehicles for CRISPR/Cas9 in both *in vitro* and *in vivo* settings in the short term.

Perspective and future directions

There are many benefits of CRISPR/Cas9 systems that can be utilized using *in vitro* laboratory engineering. However, full realization of the potential of CRISPR/Cas9 approaches will require addressing many challenges. Within the system itself, off-target cutting remains a problem. Cas9 nickases and mutants that reduce non-specific DNA binding have been engineered specifically to ameliorate this problem, though they are an imperfect solution. Extensive efforts have been made in understanding sgRNA binding and mismatch tolerance, leading to the development of several predictive software sgRNA design tools; however, our understanding of off-target effects remains poor. This is a vital area for continued study if CRISPR/Cas9 is to realize its promise.

Regarding gene cargo delivery systems, this remains the greatest obstacle for CRISPR/Cas9 use, and an all-purpose delivery method has yet to emerge. Instead, multiple methods are seen for delivering CRISPR to cells. Every method has both advantages and disadvantages, and some can be quite specific or ill-suited to certain types of delivery (e.g. delivery to cells in a flask vs. delivery to a living organism). Further, the best gene editing results with minimal off-target effects are generally obtained from delivery of the ribonucleoprotein, as opposed to plasmid DNA or mRNA. Currently, there are more options for delivery of small-molecule cargo than for the relatively large protein–nucleic acid complex. Development of new delivery approaches that enable effective RNP delivery will make a meaningful impact to the field. Still another barrier for delivery systems is ensuring that the chosen system is both safe and specific. Safety in living organisms will always be a concern, and a delivery vehicle that can target the desired cells with high-specificity will also limit off-target effects and improve safety. Additionally, it is vitally important that, especially in the case of nanoparticle carriers, long-term studies on safety of the component pieces are done. There is currently limited information available on where various components of nanoparticle delivery systems end up in the body, how long they stay there, and whether there is any long-term toxicity associated with any component.

As evident through the many examples presented in the 'Delivery Methods' portion of this review, the therapeutic potential of CRISPR/Cas9 is great. Already, much has been published on the altering of cell line genotypes and phenotypes using this gene editing system. Work has even moved into animal models, and therapeutic effects are broad-ranging, including inhibition of viral infection, reversal of debilitating conditions such as muscular dystrophy, and elimination of tumors in cancer models. Taken together, it is easy to see the reason for so much excitement in the CRISPR field. As the technology evolves and CRISPR becomes even more mechanistically precise and can be delivered with ever-increasing precision, its therapeutic potential will continue to rise.

Importantly, the CRISPR field is evolving at an incredible pace, with the number of peer-reviewed scientific papers with the term CRISPR in the title or abstract increasing by 1,453% since 2011. The outlook for the technology, therefore, is certainly positive, and we expect that with the large number of researchers from divergent fields now focusing on this system, any limitations will eventually be addressed and solved. Indeed, CRISPR is even beginning to make its way into modern-day popular culture, with casual references in multiple media formats. Truly, CRISPR is the new face of modern genetic engineering.

Acknowledgements

The authors would like to thank Raga Krishnakumar for her insightful comments and Kimberly Butler for her contributions to the 'Emerging Delivery Technologies' section.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Laboratory Directed Research and Development program at Sandia National Laboratories, a multi-mission laboratory managed and operated by National Technology and Engineering Solutions of Sandia, LLC, a wholly owned subsidiary of Honeywell International, Inc., for the US Department of Energy's National Nuclear Security Administration under contract [DE-NA0003525]. This paper describes objective technical results and analysis. Any subjective views or opinions that might be expressed in the paper do not necessarily represent the views of the US Department of Energy or the United States Government.

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References

Ahi YS, Bangari DS, Mittal SK. (2011). Adenoviral vector immunity: its implications and circumvention strategies. CGT 11:307–20.

Anders C, Bargsten K, Jinek M. (2016). Structural plasticity of PAM recognition by engineered variants of the RNA-guided endonuclease Cas9. Mol Cell 61:895–902.

Anders C, Niewoehner O, Duerst A, Jinek M. (2014). Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 513:569–73.

Axford DS, Morris DP, McMurry JL. (2017). Cell penetrating peptide-mediated nuclear delivery of Cas9 to enhance the utility of CRISPR/Cas genome editing. FASEB J 31:909.4.

Barrangou R, Fremaux C, Deveau H, et al. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709–12.

Bates K, Kostarelos K. (2013). Carbon nanotubes as vectors for gene therapy: past achievements, present challenges and future goals. Adv Drug Deliv Rev 65:2023–33.

Bestor TH. (2000). Gene silencing as a threat to the success of gene therapy. J Clin Invest 105:409–11.

Bibikova M, Beumer K, Trautman JK, Carroll D. (2003). Enhancing gene targeting with designed zinc finger nucleases. Science 300:764.

Bibikova M, Golic M, Golic K, Carroll GD. (2002). Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases. Genetics 161:1169–75.

Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 151:2551–61.

Bolukbasi MF, Gupta A, Oikemus S, et al. (2015). DNA-binding-domain fusions enhance the targeting range and precision of Cas9. Nat Methods 12:1150–6.

Bonamassa B, Hai L, Liu D. (2011). Hydrodynamic gene delivery and its applications in pharmaceutical research. Pharm Res 28:694–701.

Brito JL, Davies FE, Gonzalez D, Morgan GJ. (2008). Streptolysin-O reversible permeabilisation is an effective method to transfect siRNAs into myeloma cells. J Immunol Methods 333:147–55.

Brouns SJ, Jore MM, Lundgren M, et al. (2008). Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321:960–4.

Capecchi MR. (1989). Altering the genome by homologous recombination. Science 244:1288–92.

Carroll D. (2011). Genome engineering with zinc-finger nucleases. Genetics 188:773–82.

Carroll KJ, Makarewich CA, McAnally J, et al. (2016). A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. Proc Natl Acad Sci USA 113:338–43.

Cavazzana M, Six E, Lagresle-Peyrou C, et al. (2016). Gene therapy for X-linked severe combined immunodeficiency: where do we stand? Hum Gene Ther 27:108–16.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288:669–72.

Cermak T, Doyle EL, Christian M, et al. (2011). Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 39:e82.

Check E. (2002). A tragic setback. Nature 420:116–8.

Chen X, Goncalves MA. (2016). Engineered viruses as genome editing devices. Mol Ther 24:447–57.

Cheng R, Peng J, Yan Y, et al. (2014). Efficient gene editing in adult mouse livers via adenoviral delivery of CRISPR/Cas9. FEBS Lett 588:3954–8.

Chen S, Lee B, Lee AY, et al. (2016). Highly efficient mouse genome editing by CRISPR ribonucleoprotein electroporation of zygotes. J Biol Chem 291:14457–67.

Chen F, Pruett-Miller SM, Huang Y, et al. (2011). High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. Nat Methods 8:753–5.

Chew WL, Tabebordbar M, Cheng JK, et al. (2016). A multifunctional AAV-CRISPR-Cas9 and its host response. Nat Methods 13:868–74.

Choi PS, Meyerson M. (2014). Targeted genomic rearrangements using CRISPR/Cas technology. Nat Commun 5:3728.

Cho SW, Kim S, Kim Y, et al. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res 24:132–41.

Cho SW, Kim S, Kim JM, Kim JS. (2013). Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 31:230–2.

Christian M, Cermak T, Doyle EL, et al. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186:757–61.

Chu VT, Weber T, Wefers B, et al. (2015). Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotechnol 33:543–8.

Chuang CK, Chen CH, Huang CL, et al. (2017). Generation of GGTA1 mutant pigs by direct pronuclear microinjection of CRISPR/Cas9 plasmid vectors. Anim Biotechnol 28:174–81.

Chylinski K, Le Rhun A, Charpentier E. (2013). The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems. RNA Biol 10:726–37.

Chylinski K, Makarova KS, Charpentier E, Koonin EV. (2014). Classification and evolution of type II CRISPR-Cas systems. Nucleic Acids Res 42:6091–105.

Cong L, Ran FA, Cox D, et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–23.

Cox DB, Platt RJ, Zhang F. (2015). Therapeutic genome editing: prospects and challenges. Nat Med 21:121–31.

Cradick TJ, Fine EJ, Antico CJ, Bao G. (2013). CRISPR/Cas9 systems targeting beta-globin and CCR5 genes have substantial off-target activity. Nucleic Acids Res 41:9584–92.

Crispo M, Mulet AP, Tesson L, et al. (2015). Efficient generation of myostatin knock-out sheep using CRISPR/Cas9 technology and microinjection into zygotes. PLoS One 10:e0136690.

D'Astolfo DS, Pagliero RJ, Pras A, et al. (2015). Efficient intracellular delivery of native proteins. Cell 161:674–690.

Daya S, Berns KI. (2008). Gene therapy using adeno-associated virus vectors. Clin Microbiol Rev 21:583–93.

Deltcheva E, Chylinski K, Sharma CM, et al. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471:602–7.

Deng W, Shi X, Tjian R, et al. (2015). CASFISH: CRISPR/Cas9-mediated in situ labeling of genomic loci in fixed cells. Proc Natl Acad Sci USA 112:11870–5.

Deyle DR, Russell DW. (2009). Adeno-associated virus vector integration. Curr Opin Mol Ther 11:442–7.

Ding Q, Strong A, Patel KM, et al. (2014). Permanent alteration of PCSK9 with *in vivo* CRISPR-Cas9 genome editing. Circ Res 115:488–92.

Doench JG, Hartenian E, Graham DB, et al. (2014). Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nat Biotechnol 32:1262–7.

Dong C, Qu L, Wang H, et al. (2015). Targeting hepatitis B virus cccDNA by CRISPR/Cas9 nuclease efficiently inhibits viral replication. Antiviral Res 118:110–7.

Dow LE, Fisher J, O'Rourke KP, et al. (2015). Inducible *in vivo* genome editing with CRISPR-Cas9. Nat Biotechnol 33:390–4.

Doyon Y, Vo TD, Mendel MC, et al. (2011). Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. Nat Methods 8:74–9.

Du X, Shi B, Tang Y, et al. (2014). Label-free dendrimer-like silica nanohybrids for traceable and controlled gene delivery. Biomaterials 35:5580–90.

Durfee PN, Lin YS, Dunphy DR, et al. (2016). Mesoporous silica nanoparticle-supported lipid bilayers (protocells) for active targeting and delivery to individual leukemia cells. ACS Nano 10:8325–45.

Dykman LA, Khlebtsov NG. (2017). Immunological properties of gold nanoparticles. Chem Sci 8:1719–35.

Ebina H, Misawa N, Kanemura Y, Koyanagi Y. (2013). Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Sci Rep 3:2510.

Esvelt KM, Mali P, Braff JL, et al. (2013). Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. Nat Methods 10:1116–21.

Findlay GM, Boyle EA, Hause RJ, et al. (2014). Saturation editing of genomic regions by multiplex homology-directed repair. Nature 513:120–3.

Follenzi A, Santambrogio L, Annoni A. (2007). Immune responses to lentiviral vectors. Curr Gene Ther 7:306–15.

Fu Y, Foden JA, Khayter C, et al. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 31:822–6.

Gabriel R, Lombardo A, Arens A, et al. (2011). An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotechnol 29:816–23.

Garrett RA, Shah SA, Erdmann S, et al. (2015). CRISPR-Cas adaptive immune systems of the sulfolobales: unravelling their complexity and diversity. Life (Basel, Switzerland) 5:783–817.

Gaspar HB, Cooray S, Gilmour KC, et al. (2011). Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. Sci Translational Med 3:97ra79.

Gaspar HB, Parsley KL, Howe S, et al. (2004). Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet (London, England) 364:2181–7.

Gaudelli NM, Komor AC, Rees HA, et al. (2017). Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. Nature 551:464–71.

Gilbert LA, Horlbeck MA, Adamson B, et al. (2014). Genome-scale CRISPR-mediated control of gene repression and activation. Cell 159:647–61.

Gonzalez Porras MA, Durfee PN, Gregory AM, et al. (2016). A novel approach for targeted delivery to motoneurons using cholera toxin-B modified protocells. J Neurosci Methods 273:160–74.

Guan Y, Ma Y, Li Q, et al. (2016). CRISPR/Cas9-mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse. EMBO Mol Med 8:477–88.

Guilinger JP, Thompson DB, Liu DR. (2014). Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. Nat Biotechnol 32:577–82.

Gupta RM, Musunuru K. (2014). Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. J Clin Invest 124:4154–61.

Hacein-Bey-Abina S, Hauer J, Lim A, et al. (2010). Efficacy of gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 363:355–64.

Hacein-Bey-Abina S, Le Deist F, Carlier F, et al. (2002). Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. N Engl J Med 346:1185–93.

Haeussler M, Schonig K, Eckert H, et al. (2016). Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol 17:148.

Hashimoto M, Takemoto T. (2015). Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing. Sci Rep 5:11315.

Heckl D, Kowalczyk MS, Yudovich D, et al. (2014). Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. Nat Biotechnol 32:941–6.

Hefferin ML, Tomkinson AE. (2005). Mechanism of DNA double-strand break repair by non-homologous end joining. DNA Repair (Amst) 4:639–48.

Hilton IB, D'Ippolito AM, Vockley CM, et al. (2015). Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nat Biotechnol 33:510–7.

Hirano S, Nishimasu H, Ishitani R, Nureki O. (2016). Structural basis for the altered PAM specificities of engineered CRISPR-Cas9. Mol Cell 61:886–94.

Horii T, Arai Y, Yamazaki M, et al. (2014). Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. Sci Rep 4:4513.

Horii T, Tamura D, Morita S, et al. (2013). Generation of an ICF syndrome model by efficient genome editing of human induced pluripotent stem cells using the CRISPR system. IJMS 14:19774–81.

Hou Z, Zhang Y, Propson NE, et al. (2013). Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. Proc Natl Acad Sci 110:15644–9.

Hsu PD, Scott DA, Weinstein JA, et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31:827–32.

Hung SS, Chrysostomou V, Li F, et al. (2016). AAV-mediated CRISPR/Cas gene editing of retinal cells *in vivo*. Invest Ophthalmol Vis Sci 57:3470–6.

Ishino Y, Shinagawa H, Makino K, et al. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol 169:5429–33.

Jansen R, Embden JD, Gaastra W, Schouls LM. (2002). Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol 43:1565–75.

Jiang W, Bikard D, Cox D, et al. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 31:233–9.

Jinek M, Chylinski K, Fonfara I, et al. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–21.

Jinek M, Jiang F, Taylor DW, et al. (2014). Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 343:1247997.

Joung JK, Sander JD. (2013). TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 14:49–55.

Kabadi AM, Ousterout DG, Hilton IB, Gersbach CA. (2014). Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. Nucleic Acids Res 42:e147.

Kaiser J. (2003). Gene therapy. Seeking the cause of induced leukemias in X-SCID trial. Science 299:495.

Kennedy EM, Kornepati AV, Goldstein M, et al. (2014). Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. J Virol 88:11965–72.

Kim S, Kim D, Cho SW, et al. (2014). Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res 24:1012–9.

Kleinstiver BP, Pattanayak V, Prew MS, et al. (2016). High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 529:490–5.

Kleinstiver BP, Prew MS, Tsai SQ, et al. (2015). Broadening the targeting range of *Staphylococcus aureus* CRISPR-Cas9 by modifying PAM recognition. Nat Biotechnol 33:1293–8.

Kleinstiver BP, Prew MS, Tsai SQ, et al. (2015). Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523:481–5.

Kogure K, Moriguchi R, Sasaki K, et al. (2004). Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method. J Control Release 98:317–23.

Koike-Yusa H, Li Y, Tan EP, et al. (2014). Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nat Biotechnol 32:267–73.

Konermann S, Brigham MD, Trevino AE, et al. (2015). Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 517:583–8.

LaFountaine JS, Fathe K, Smyth HD. (2015). Delivery and therapeutic applications of gene editing technologies ZFNs, TALENs, and CRISPR/Cas9. Int J Pharm 494:180–94.

Lander ES. (2016). The heroes of CRISPR. Cell 164:18–28.

Ledford H. (2016). CRISPR: gene editing is just the beginning. Nature 531:156–9.

Lee K, Conboy M, Park HM, et al. (2017). Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nat Biomed Eng 1:889–901.

Liang X, Potter J, Kumar S, et al. (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. J Biotechnol 208:44–53.

Li C, Guan X, Du T, et al. (2015). Inhibition of HIV-1 infection of primary CD4+ T-cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9. J Gen Virol 96:2381–93.

Li T, Huang S, Jiang WZ, et al. (2011). TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. Nucleic Acids Res 39:359–72.

Lin S, Staahl BT, Alla RK, Doudna JA. (2014). Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Elife 3:e04766.

Lin SR, Yang HC, Kuo YT, et al. (2014). The CRISPR/Cas9 system facilitates clearance of the intrahepatic HBV templates *in vivo*. Mol Ther Nucleic Acids 3:e186.

Liu J, Stace-Naughton A, Jiang X, Brinker CJ. (2009). Porous nanoparticle supported lipid bilayers (protocells) as delivery vehicles. J Am Chem Soc 131:1354–5.

Liu Y, Zeng Y, Liu L, et al. (2014). Synthesizing AND gate genetic circuits based on CRISPR-Cas9 for identification of bladder cancer cells. Nat Commun 5:5393.

Long C, Amoasii L, Mireault AA, et al. (2016). Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. Science 351:400–3.

Long C, McAnally JR, Shelton JM, et al. (2014). Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. Science 345:1184–8.

Luo GF, Chen WH, Liu Y, et al. (2014). Multifunctional enveloped mesoporous silica nanoparticles for subcellular co-delivery of drug and therapeutic peptide. Sci Rep 4:6064.

Luo D, Saltzman WM. (2000). Enhancement of transfection by physical concentration of DNA at the cell surface. Nat Biotechnol 18:893–5.

Mackowiak SA, Schmidt A, Weiss V, et al. (2013). Targeted drug delivery in cancer cells with red-light photoactivated mesoporous silica nanoparticles. Nano Lett 13:2576–83.

Ma H, Dang Y, Wu Y, et al. (2015). A CRISPR-based screen identifies genes essential for West-Nile-Virus-induced cell death. Cell Rep 12:673–83.
Maddalo D, Manchado E, Concepcion CP, et al. (2014). In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. Nature 516:423–7.
Maggio I, Stefanucci L, Janssen JM, et al. (2016). Selection-free gene repair after adenoviral vector transduction of designer nucleases: rescue of dystrophin synthesis in DMD muscle cell populations. Nucleic Acids Res 44:1449–70.
Makarova KS, Haft DH, Barrangou R, et al. (2011). Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9:467–77.
Makarova KS, Koonin EV. (2015). Annotation and classification of CRISPR-Cas systems. Methods Mol Biol 1311:47–75.
Makarova KS, Wolf YI, Alkhnbashi OS, et al. (2015). An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol 13:722–36.
Mali P, Aach J, Stranges PB, et al. (2013). CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 31:833–8.
Mali P, Yang L, Esvelt KM, et al. (2013). RNA-guided human genome engineering via Cas9. Science 339:823–6.
Ma H, Naseri A, Reyes-Gutierrez P, et al. (2015). Multicolor CRISPR labeling of chromosomal loci in human cells. Proc Natl Acad Sci USA 112:3002–7.
Marshall E. (1999). Gene therapy death prompts review of adenovirus vector. Science 286:2244–5.
Maruyama T, Dougan SK, Truttmann MC, et al. (2015). Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol 33:538–42.
Ma Y, Shen B, Zhang X, et al. (2014). Heritable multiplex genetic engineering in rats using CRISPR/Cas9. PLoS One 9:e89413.
Matano M, Date S, Shimokawa M, et al. (2015). Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. Nat Med 21:256–62.
Ma Y, Zhang L, Huang X. (2014). Genome modification by CRISPR/Cas9. FEBS J 281:5186–93.
Miller JC, Holmes MC, Wang J, et al. (2007). An improved zinc-finger nuclease architecture for highly specific genome editing. Nat Biotechnol 25:778–85.
Miller JC, Tan S, Qiao G, et al. (2011). A TALE nuclease architecture for efficient genome editing. Nat Biotechnol 29:143–8.
Miller JB, Zhang S, Kos P, et al. (2017). Non-viral CRISPR/Cas gene editing *in vitro* and *in vivo* enabled by synthetic nanoparticle co-delivery of Cas9 mRNA and sgRNA. Angew Chem Int Ed Engl 56:1059–63.
Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol 60:174–82.
Mojica FJ, Diez-Villasenor C, Soria E, Juez G. (2000). Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Mol Microbiol 36:244–6.
Mojica FJ, Juez G, Rodriguez-Valera F. (1993). Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites. Mol Microbiol 9:613–21.
Moreno-Mateos MA, Vejnar CE, Beaudoin JD, et al. (2015). CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. Nat Methods 12:982–8.
Mout R, Ray M, Yesilbag Tonga G, et al. (2017). Direct cytosolic delivery of CRISPR/Cas9-ribonucleoprotein for efficient gene editing. ACS Nano 11:2452–8.
Mussolino C, Alzubi J, Fine EJ, et al. (2014). TALENs facilitate targeted genome editing in human cells with high specificity and low cytotoxicity. Nucleic Acids Res 42:6762–73.
Nakagawa Y, Sakuma T, Sakamoto T, et al. (2015). Production of knockout mice by DNA microinjection of various CRISPR/Cas9 vectors into freeze-thawed fertilized oocytes. BMC Biotechnol 15:33.
Nakamura T, Akita H, Yamada Y, et al. (2012). A multifunctional envelope-type nanodevice for use in nanomedicine: concept and applications. Acc Chem Res 45:1113–21.
Naldini L, Blomer U, Gallay P, et al. (1996). *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263–7.
Nelson CE, Hakim CH, Ousterout DG, et al. (2016). In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. Science 351:403–7.
Nihongaki Y, Kawano F, Nakajima T, Sato M. (2015). Photoactivatable CRISPR-Cas9 for optogenetic genome editing. Nat Biotechnol 33:755–60.
NIH Report. (2002). Assessment of adenoviral vector safety and toxicity: report of the National Institutes of Health Recombinant DNA Advisory Committee. Hum Gene Ther 13:3–13.
Nishimasu H, Cong L, Yan WX, et al. (2015). Crystal structure of *Staphylococcus aureus* Cas9. Cell 162:1113–26.
Niu Y, Shen B, Cui Y, et al. (2014). Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156:836–43.
Ousterout DG, Kabadi AM, Thakore PI, et al. (2015). Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. Nat Commun 6:6244.
Papapetrou EP, Schambach A. (2016). Gene insertion into genomic safe harbors for human gene therapy. Mol Ther 24:678–84.
Paquet D, Kwart D, Chen A, et al. (2016). Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. Nature 533:125–9.
Pattanayak V, Lin S, Guilinger JP, et al. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nat Biotechnol 31:839–43.
Pattanayak V, Ramirez CL, Joung JK, Liu DR. (2011). Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. Nat Methods 8:765–70.
Platt RJ, Chen S, Zhou Y, et al. (2014). CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 159:440–55.
Polstein LR, Gersbach CA. (2015). A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. Nat Chem Biol 11:198–200.
Pourcel C, Salvignol G, Vergnaud G. (2005). CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 151:653–63.
Qi LS, Larson MH, Gilbert LA, et al. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152:1173–83.
Qin W, Dion SL, Kutny PM, et al. (2015). Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease. Genetics 200:423–30.
Radecke S, Radecke F, Cathomen T, Schwarz K. (2010). Zinc-finger nuclease-induced gene repair with oligodeoxynucleotides: wanted and unwanted target locus modifications. Mol Ther 18:743–53.
Raitskin O, Patron NJ. (2016). Multi-gene engineering in plants with RNA-guided Cas9 nuclease. Curr Opin Biotechnol 37:69–75.
Ramakrishna S, Kwaku Dad AB, Beloor J, et al. (2014). Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome Res 24:1020–7.
Ramirez CL, Foley JE, Wright DA, et al. (2008). Unexpected failure rates for modular assembly of engineered zinc fingers. Nat Methods 5:374–5.
Ran FA, Cong L, Yan WX, et al. (2015). In vivo genome editing using *Staphylococcus aureus* Cas9. Nature 520:186–91.
Ran FA, Hsu PD, Lin CY, et al. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154:1380–9.
Raveux A, Vandormael-Pournin S, Cohen-Tannoudji M. (2017). Optimization of the production of knock-in alleles by CRISPR/Cas9 microinjection into the mouse zygote. Sci Rep 7:42661.
Reyon D, Tsai SQ, Khayter C, et al. (2012). FLASH assembly of TALENs for high-throughput genome editing. Nat Biotechnol 30:460–5.

Robert F, Barbeau M, Ethier S, et al. (2015). Pharmacological inhibition of DNA-PK stimulates Cas9-mediated genome editing. Genome Med 7:93.

Roehm PC, Shekarabi M, Wollebo HS, et al. (2016). Inhibition of HSV-1 replication by gene editing strategy. Sci Rep 6:23146.

Ross J. (1995). mRNA stability in mammalian cells. Microbiol Rev 59:423–50.

Rouet P, Smih F, Jasin M. (1994). Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. Proc Natl Acad Sci USA 91:6064–8.

Sakuma T, Nishikawa A, Kume S, et al. (2014). Multiplex genome engineering in human cells using all-in-one CRISPR/Cas9 vector system. Sci Rep 4:5400.

Samulski RJ, Muzyczka N. (2014). AAV-mediated gene therapy for research and therapeutic purposes. Annu Rev Virol 1:427–51.

Sato M, Koriyama M, Watanabe S, et al. (2015). Direct injection of CRISPR/Cas9-related mRNA into cytoplasm of parthenogenetically activated porcine oocytes causes frequent mosaicism for indel mutations. Ijms 16:17838–56.

Schumann K, Lin S, Boyer E, et al. (2015). Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. Proc Natl Acad Sci USA 112:10437–42.

Schwank G, Koo BK, Sasselli V, et al. (2013). Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell Stem Cell 13:653–8.

Shalem O, Sanjana NE, Hartenian E, et al. (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 343:84–7.

Shen B, Zhang J, Wu H, et al. (2013). Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell Res 23:720–3.

Shmakov S, Abudayyeh OO, Makarova KS, et al. (2015). Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. Mol Cell 60:385–97.

Sierig G, Cywes C, Wessels MR, Ashbaugh CD. (2003). Cytotoxic effects of streptolysin o and streptolysin s enhance the virulence of poorly encapsulated group a streptococci. Infect Immun 71:446–55.

Slaymaker IM, Gao L, Zetsche B, et al. (2016). Rationally engineered Cas9 nucleases with improved specificity. Science 351:84–8.

Soldner F, Laganiere J, Cheng AW, et al. (2011). Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. Cell 146:318–31.

Srivastava M, Nambiar M, Sharma S, et al. (2012). An inhibitor of nonhomologous end-joining abrogates double-strand break repair and impedes cancer progression. Cell 151:1474–87.

Sternberg SH, Redding S, Jinek M, et al. (2014). DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature 507:62–7.

Strutt SC, Torrez RM, Kaya E, et al. (2018). RNA-dependent RNA targeting by CRISPR-Cas9. Elife 7.

Su J, Sun H, Meng Q, et al. (2017). Enhanced blood suspensibility and laser-activated tumor-specific drug release of theranostic mesoporous silica nanoparticles by functionalizing with erythrocyte membranes. Theranostics 7:523–37.

Suda T, Gao X, Stolz DB, Liu D. (2007). Structural impact of hydrodynamic injection on mouse liver. Gene Ther 14:129–37.

Sun W, Jiang T, Lu Y, et al. (2014). Cocoon-like self-degradable DNA nanoclew for anticancer drug delivery. J Am Chem Soc 136:14722–5.

Sun W, Ji W, Hall JM, et al. (2015). Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. Angew Chem Int Ed Engl 54:12029–33.

Swiech L, Heidenreich M, Banerjee A, et al. (2015). *In vivo* interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nat Biotechnol 33:102–6.

Szczelkun MD, Tikhomirova MS, Sinkunas T, et al. (2014). Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. Proc Natl Acad Sci USA 111:9798–803.

Tabebordbar M, Zhu K, Cheng JKW, et al. (2016). In vivo gene editing in dystrophic mouse muscle and muscle stem cells. Science 351:407–11.

Takata M, Sasaki MS, Sonoda E, et al. (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J 17:5497–508.

Tanenbaum ME, Gilbert LA, Qi LS, et al. (2014). A protein-tagging system for signal amplification in gene expression and fluorescence imaging. Cell 159:635–46.

Teng KW, Ishitsuka Y, Ren P, et al. (2017). Labeling proteins inside living cells using external fluorophores for fluorescence microscopy. Elife 6:e25460.

Thomas CE, Ehrhardt A, Kay MA. (2003). Progress and problems with the use of viral vectors for gene therapy. Nat Rev Genet 4:346–58.

Tomkinson AE, Howes TR, Wiest NE. (2013). DNA ligases as therapeutic targets. Translational Cancer Res 2:1219.

Truong DJ, Kuhner K, Kuhn R, et al. (2015). Development of an intein-mediated split-Cas9 system for gene therapy. Nucleic Acids Res 43:6450–8.

Tsai SQ, Wyvekens N, Khayter C, et al. (2014). Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol 32:569–76.

Urnov FD, Rebar EJ, Holmes MC, et al. (2010). Genome editing with engineered zinc finger nucleases. Nat Rev Genet 11:636–46.

van der Oost J, Jore MM, Westra ER, et al. (2009). CRISPR-based adaptive and heritable immunity in prokaryotes. Trends Biochem Sci 34:401–7.

van Soolingen D, de Haas PE, Hermans PW, et al. (1993). Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of Mycobacterium tuberculosis. J Clin Microbiol 31:1987–95.

Vartak SV, Raghavan SC. (2015). Inhibition of nonhomologous end joining to increase the specificity of CRISPR/Cas9 genome editing. FEBS J 282:4289–94.

Voets O, Tielen F, Elstak E, et al. (2017). Highly efficient gene inactivation by adenoviral CRISPR/Cas9 in human primary cells. PLoS One 12:e0182974.

Vora S, Tuttle M, Cheng J, Church G. (2016). Next stop for the CRISPR revolution: RNA-guided epigenetic regulators. FEBS J 283:3181–93.

Walev I, Bhakdi SC, Hofmann F, et al. (2001). Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. Proc Natl Acad Sci U S A 98:3185–90.

Wang D, Huang J, Wang X, et al. (2013). The eradication of breast cancer cells and stem cells by 8-hydroxyquinoline-loaded hyaluronan modified mesoporous silica nanoparticle-supported lipid bilayers containing docetaxel. Biomaterials 34:7662–73.

Wang D, Mou H, Li S, et al. (2015). Adenovirus-mediated somatic genome editing of Pten by CRISPR/Cas9 in mouse liver in spite of Cas9-specific immune responses. Hum Gene Ther 26:432–42.

Wang J, Quake SR. (2014). RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection. Proc Natl Acad Sci 111:13157–62.

Wang T, Wei JJ, Sabatini DM, Lander ES. (2014). Genetic screens in human cells using the CRISPR-Cas9 system. Science 343:80–4.

Wang H, Yang H, Shivalila CS, et al. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153:910–8.

Wang M, Zuris JA, Meng F, et al. (2016). Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles. Proc Natl Acad Sci USA 113:2868–73.

Weinstock DM, Jasin M. (2006). Alternative pathways for the repair of RAG-induced DNA breaks. Mol Cell Biol 26:131–9.

Wiedenheft B, Lander GC, Zhou K, et al. (2011). Structures of the RNA-guided surveillance complex from a bacterial immune system. Nature 477:486–9.

Wolfe SA, Nekludova L, Pabo CO. (2000). DNA recognition by Cys2His2 zinc finger proteins. Annu Rev Biophys Biomol Struct 29:183–212.

Wright AV, Nunez JK, Doudna JA. (2016). Biology and applications of CRISPR systems: Harnessing Nature's Toolbox for genome engineering. Cell 164:29–44.

Wu Y, Liang D, Wang Y, et al. (2013). Correction of a genetic disease in mouse via use of CRISPR-Cas9. Cell Stem Cell 13:659–62.

Wu Z, Yang H, Colosi P. (2010). Effect of genome size on AAV vector packaging. Mol Ther 18:80–6.

Wu Y, Zhou H, Fan X, et al. (2015). Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. Cell Res 25:67–79.

Xu H, Xiao T, Chen CH, et al. (2015). Sequence determinants of improved CRISPR sgRNA design. Genome Res 25:1147–57.

Xue W, Chen S, Yin H, et al. (2014). CRISPR-mediated direct mutation of cancer genes in the mouse liver. Nature 514:380–4.

Yang H, Wang H, Shivalila CS, et al. (2013). One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154:1370–9.

Yao S, He Z, Chen C. (2015). CRISPR/Cas9-mediated genome editing of epigenetic factors for cancer therapy. Hum Gene Ther 26:463–71.

Ye L, Wang J, Beyer AI, et al. (2014). Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Delta32 mutation confers resistance to HIV infection. Proc Natl Acad Sci USA 111:9591–6.

Yin H, Song CQ, Dorkin JR, et al. (2016). Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nat Biotechnol 34:328–33.

Yin H, Xue W, Chen S, et al. (2014). Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nat Biotechnol 32:551–3.

Yu C, Liu Y, Ma T, et al. (2015). Small molecules enhance CRISPR genome editing in pluripotent stem cells. Cell Stem Cell 16:142–7.

Zhang D, Li Z, Li JF. (2016). Targeted gene manipulation in plants using the CRISPR/Cas technology. J Genet Genomics = Yi Chuan Xue Bao 43:251–62.

Zhang R, Miner JJ, Gorman MJ, et al. (2016). A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. Nature 535:164–8.

Zhang Y, Ma X, Xie X, Liu Y-G. (2017). CRISPR/Cas9-based genome editing in plants. Prog Mol Biol Transl Sci 149:133–50.

Zhang Z, Zhang S, Huang X, et al. (2013). Rapid assembly of customized TALENs into multiple delivery systems. PLoS One 8:e80281.

Zhen S, Hua L, Liu YH, et al. (2015). Harnessing the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas9 system to disrupt the hepatitis B virus. Gene Ther 22:404–12.

Zhou Y, Zhu S, Cai C, et al. (2014). High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. Nature 509:487–91.

Zhu H, Lau CH, Goh SL, et al. (2013). Baculoviral transduction facilitates TALEN-mediated targeted transgene integration and Cre/LoxP cassette exchange in human-induced pluripotent stem cells. Nucleic Acids Res 41:e180.

Zuckermann M, Hovestadt V, Knobbe-Thomsen CB, et al. (2015). Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. Nat Commun 6:7391.

Zuris JA, Thompson DB, Shu Y, et al. (2015). Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing *in vitro* and *in vivo*. Nat Biotechnol 33:73–80.

Journal of Controlled Release xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review article

Single-cell transfection technologies for cell therapies and gene editing

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ARTICLE INFO

Keywords:
Single-cell
Transfection
Intracellular delivery
Cell therapies
Gene editing
Electroporation
Optoporation
Cell squeezing
Microfluidic vortex shedding
Microinjection
Good manufacturing practices

ABSTRACT

Advances in gene editing and cell therapies have recently led to outstanding clinical successes. However, the lack of a cost-effective manufacturing process prevents the democratization of these innovative medical tools. Due to the common use of viral vectors, the step of transfection in which cells are engineered to gain new functions, is a major bottleneck in making safe and affordable cell products. A promising opportunity lies in Single-Cell Transfection Technologies (SCTTs). SCTTs have demonstrated higher efficiency, safety and scalability than conventional transfection methods. They can also feature unique abilities such as substantial dosage control over the cargo delivery, single-cell addressability and integration in microdevices comprising multiple monitoring modalities. Unfortunately, the potential of SCTTs is not fully appreciated: they are most often restricted to research settings with little adoption in clinical settings. To encourage their adoption, we review and compare recent developments in SCTTs, and how they can enable selected clinical applications. To help bridge the gap between fundamental research and its translation to the clinic, we also describe how Good Manufacturing Practices (GMP) can be integrated in the design of SCTTs.

1. Introduction

Transfection, the intracellular delivery of nucleic acids and proteins, is a crucial part in the development of cell-based therapies. Cell-based therapies are innovative approaches making use of cells that have been genetically engineered to replace defective organ functions, treat diseases or model physiological and pathological behaviors in vitro. The most promising example so far is Chimeric Antigen Receptor (CAR) T cell immunotherapy: by introducing a tumor-targeting receptor in a cancer patient's T cells, they are turned into a "living cancer drug", essentially reprogramming the patient's immune system. CAR T cell therapies have shown tremendous success against B cell malignancies and two commercial products have recently been approved by the Food and Drug Administration for the treatment of lymphoma [1].

The clinical applications of genetically engineered cells are numerous. CAR T cells have also been harnessed to combat HIV. Other immune cells that have been reprogrammed to treat cancer include natural killer cells and macrophages [1]. Gene editing of stem cells offers the possibility to cure monogenetic diseases and generate a source of replacement tissues. Induced Pluripotent Stem Cells (iPSC) can generate more predictive, humanized disease models for patient stratification and drug discovery and testing, so-called "clinical trials in a dish". There is no doubt that modified human cells will take an important place in tomorrow's pharmaceutical toolbox [2–8].

Conventional transfection methods used to engineer cells have, however, significant limitations. The use of cuvette-based electroporation, cationic liposomes and viral vectors in scaled up manufacturing of cell products is hindered by low cell viability and delivery efficiency, virtually inexistent spatiotemporal resolution and labor-intensive, variability-prone manual operation. Manufacturing capabilities for clinical-grade viral vectors are inadequate, resulting in high prices and restricted availability [1,9].The use of viral vectors also introduces safety risks linked with insertional mutagenesis, uncontrolled expression of gene constructs and immunogenicity [10].

Single-Cell Transfection Technologies (SCTTs) are a promising alternative. We define them here as technologies capable of inducing

Abbreviation: Single-cell transfection technology, SCTT; Good Manufacturing Practices, GMP; Chimeric antigen receptor, CAR; Induced pluripotent stem cells, iPSC; Dendritic cell, DC; Antigen presenting cell, APC; Human leukocyte antigen, HLA; T cell receptor, TCR; Cas9-guide RNA ribonucleoprotein complex, RNP; Organ-on-Chips, OoC; Microelectrode arrays, MEA; Mouse Embryonic Fibroblast, MEF.

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https://doi.org/10.1016/j.jconrel.2020.10.068
Received 14 July 2020; Received in revised form 30 October 2020; Accepted 31 October 2020
Available online 5 November 2020

membrane permeation through actuation at the single-cell level, allowing for a more efficient and less disruptive transfection. Moreover, they can feature substantial dosage control over the cargo delivery, single-cell addressability and integration in microdevices comprising multiple monitoring modalities.

This work presents a critical overview of the latest advances in SCTTs. We first list a selection of clinical applications and their requirements for more sophisticated transfection methods. Second, we address the most promising SCTTs published in the literature during the last five to ten years and explain how we expect their unique properties to drastically benefit, if not enable, the selected clinical applications. Finally, as we aim at fostering the further development of SCTTs and, most importantly, their translation toward clinical applications, we will discuss different aspects of the Good Manufacturing Practices (GMP) that govern the commercialization of safe and qualitative medical products.

2. Applications in cell-based medicine

2.1. From fundamental research to clinical applications

In this section, we highlight clinical applications whose key needs are not optimally met by conventional transfection methods. We argue that manufacturing of cell-based therapies, with an emphasis on immunotherapy, gene editing, iPSC technology, and personalized disease models, will be drastically improved by advanced transfection technologies, in particular SCTTs.

2.2. Immunotherapies

In the immunotherapy field, CAR T cell therapies currently receive the most attention in clinical trials [6]. Briefly, T lymphocytes are extracted from the patient's blood and are genetically modified to express an artificial receptor (the CAR) granting antitumoral activity. Up to a few hundred million modified cells are then reinfused back in the patient to fight the cancer [9,11,129]. Usually, the average manufacturing process takes about two weeks [12]. FDA-approved CAR T cell therapies have provided ample clinical evidence for the feasibility of an approach that requires ex vivo genetic engineering. However, the cost and complexity of the manufacturing process has been blamed for slowing down wide therapy uptake. Automation and miniaturization are expected to be key in solving these critical issues. Non-viral CAR delivery has been earmarked as a crucial step, and in addition, is promising improved safety over the use of viral vectors [13].

Electroporation-mediated delivery of mRNA has been shown as a promising alternative to viral vectors. Delivery of the CAR in mRNA form offers lower cytotoxicity than plasmid transfection and poses no risk from random genomic integration [14,15]. Cells engineered with mRNA show fast, transient expression of the CAR and require repeated infusions to sustain an antitumor effect. This dosage and temporal control over the CAR activity can reduce on-target, off-tumor effects and is thought to open CAR T therapies to solid tumors. Delivering CAR-encoding mRNA into freshly extracted peripheral blood mononuclear cells obviates the need for activation and expansion steps, which can reduce treatment manufacturing to a single day, thus reducing cost and the risk of disease complication while the treatment is produced [16]. Limiting the culture time ex vivo also enhances antitumoral activity by preventing T cell exhaustion [17]. This approach is currently being investigated in a Phase 1 clinical trial by the company MaxCyte as part of their CARMA (CAR mRNA) program, based on CAR mRNA delivery into non-activated lymphocytes for the treatment of solid tumors (NCT03608618). Finally, delivery of a purified mRNA construct, from which double strand RNA structures have been eliminated, drastically enhances the efficacy of the engineered CAR T cells [18].

Electroporation is the method of choice to deliver nucleic acids in primary immune cells [14]. Conventional bulk electroporation, however, perturbs T cell function [19]. The low throughput and manual operation of the method are additional issues. A suitable transfection method should robustly and reproducibly deliver large nucleic acids in around a billion suspended cells while preserving their immune function. In-flow transfection methods with high throughput are therefore best suited and will be discussed below. The company Cellectis, for instance, is currently developing universal CAR T cells therapies. It uses a proprietary bulk electroporation system to deliver mRNA-encoded gene editing enzymes targeting the TCR of the processed immune cells. Cellectis has multiple ongoing clinical trials (e.g. NCT04142619).

The immunotherapy field is also interested in the modification of Antigen Presenting Cells (APC) to act as cancer vaccines. APCs are scouts of the immune system that can organize an immune response against specific antigens. In the procedure, a patient's dendritic cells (DCs, which are potent APCs) are harvested and loaded with tumor-specific antigens ex vivo, which can be peptides, proteins or nucleic acids obtained from the tumor. They are then reinfused in the patient, where they will present the antigens to immune cells and prime an immune response against the tumor [20].

Electroporation is an effective way of delivering mRNA-encoded antigens or tumor lysate in DCs [21–23]. An advantage of the method is the possibility of targeting multiple mutant, tumor-specific antigens rather than a lineage-specific protein (such as CD19 in the case of CAR T therapy for lymphoma). It would enable the treatment of solid tumors, so far difficult for CAR T cells. While DC vaccines are safer than CAR T therapies, they have not yet demonstrated a similar efficacy [24]. Other cells more abundantly present in blood than DCs, including B cells and T cells, can also perform APC functions. Peptide antigen delivery in those cells is a simple, yet promising approach to improve cancer vaccines and modulate the immune system [14,25,26].

Delivery of gene editing molecules, such as the CRISPR-Cas9 enzyme and its guide RNA, is being investigated to improve the efficiency and availability of cell therapies. In immunotherapy, knocking out immune checkpoint receptors naturally present on T cells can prevent tumor cells relying on immunoinhibitory ligands as an evasion strategy. Gene editing is also key toward developing universal, off-the-shelf CAR T cells for allogeneic adoptive cell therapies. Indeed, deriving CAR T cells from a single donor to treat multiple patients would decrease the cost per treatment [1]. To reach this goal, knocking out endogenous T Cell Receptor (TCR) and Human Leukocyte Antigen (HLA) on allogeneic CAR T cells have been used as strategies to limit graft-versus-host disease and transplant rejection, respectively [27]. Gene editing is also applied to blood cells to treat other genetic diseases. For instance, transplantation of autologous hematopoietic stem cells, in which a single gene has been corrected, could cure inherited monogenic blood diseases such as sickle cell anemia [28].

The main obstacle against the clinical use of CRISPR-Cas9 technology lies in the off-target effects associated with high cellular concentrations of Cas9-guide RNA ribonucleoprotein complex (RNP) that arises from uncontrolled expression of gene constructs encoding the nuclease and its guide RNA. The transient, dosage-controlled intracellular delivery of the RNP, rather than nucleic acids, can minimize these effects [29]. Moreover, the avoidance of a DNA vector eliminates risks of genomic integration and the associated safety concerns. Several SCTTs offer such dosage control and are discussed below.

2.3. Induced pluripotent stem cells

Over a decade ago, researchers demonstrated that the introduction of four selected transcription factors in terminally differentiated somatic cells could reprogram them to gain embryonic stem cell-like pluripotency and proliferative potential [30]. Those induced Pluripotent Stem Cells (iPSC) have been lauded as a breakthrough in regenerative medicine and as a powerful tool for in vitro drug testing and development of personalized, more predictive disease-in-a-dish models [8]. Indeed, the collection and reprogramming of patient cells would allow for the in

vitro creation of a patient-specific tissue. Recent studies aim at using iPSC transplantations to treat spinal cord injury [31], macular degeneration and thalassemia [4]. Other promising results show, for instance, that transplantation of iPSC-derived dopaminergic neurons can alleviate symptoms in primate models of Parkinson's disease [32].

So far, iPSCs have been mostly adopted in disease modelling and drug screening [4,8,28,33], since In vitro study of patient-derived cells is a more accurate representation of processes happening in vivo in comparison to immortalized cell lines. Clinical trials in a dish are expected to give faster and more predictive pre-clinical studies of drug toxicity and efficacy [8]. Finally, iPSCs differentiated into distinct tissues could be used to create personalized disease models inside microdevices that integrate cell monitoring and stimulation modalities, so called Organ-on-Chips (OoC).

The limited understanding of the reprogramming barriers and lack of an appropriate transcription factor delivery method has resulted in low reprogramming efficiencies and prevented their adoption in the clinics [7,34]. A method that can deliver stoichiometric quantities of the different transcription factors has been identified as a key need to improve reprogramming efficiencies [35–37]. However, the different reprogramming factors are usually distributed across multiple plasmids and delivered in a series of transfection events that make dosage control difficult. Reliably delivering a controlled dose of a single, large plasmid containing each factor with optimal stoichiometry would be beneficial. Another approach, generating iPSCs through delivery of mRNAs [38] and proteins [39], is a DNA-free alternative leading to fast reprogramming. It eliminates any possibility of transgene integration in the cell's genome but may reduce control of delivered amount.

Transfection of iPSCs with lineage-specific transcription factors is a fast method to generate fully differentiated cells, compared to the weeks-long step-by-step protocols using a sequence of different culture conditions and growth factors. The shorter culture period will reduce cell heterogeneity within a cell line for more accurate analysis. Finally, an efficient way to generate a large and homogenous population of a given iPSC-derived cell type will benefit high-throughput screening of molecular compounds, which require large amounts of cells [40].

Among the most cited public health priorities, the study of neurodegenerative diseases will likely benefit most from OoC development. OoC are well suited for the investigation of neuronal networks, where pathological electrophysiological activity can be non-invasively recorded and analyzed through microelectrodes patterned on the device [41]. For instance, Wainger and colleagues have grown iPSC-derived motor neurons obtained from amyotrophic lateral sclerosis patients on a microelectrode array. Through electrophysiological monitoring, they could reveal the mechanisms underlying their hyperexcitability and identify a potential drug to treat this disease [42]. Such OoC will benefit from technologies that can "print tissues" with a physiologically relevant structure directly in the device. Through spatially resolved delivery of transcription factors in iPSCs, complex networks of different neuron types can be created, allowing for a tissue-level study of disease mechanisms.

2.4. Key needs for enabling clinical applications

From the above, it is clear that several key needs exist which are insufficiently met by current transfection technologies. Specifically, there is a demand for approaches that provide: non-viral delivery, especially for manufacturing cell therapies; delivery of non-DNA cargos such as mRNA, RNA, proteins and RNPs; robust and reproducible delivery, yielding a homogenous response from the target cells; high throughput; high efficiency and low toxicity; control of cargo dosage and stoichiometry; and spatiotemporal control of cargo delivery. Transfection technologies should also be amenable to automation, integration and miniaturization, preferably in a closed-loop configuration. Finally, they should facilitate GMP compliance of the entire manufacturing process that they are part of. In the next paragraph, we describe how SCTTs could resolve these outstanding challenges, stimulating adoption of state-of-the-art research results in the clinic.

3. SCTTs: A transition to next-generation transfection methods

Here, we present and discuss the most promising examples of SCTTs for clinical applications. SCTTs have the defining ability to reliably apply a membrane permeation stimulus through actuation at the single-cell level, rather than exposing an entire population of cells to a global stimulus. Those technologies offer higher yield, throughput, safety, versatility in cell and cargo types or scalability than conventional transfection methods. Among other features, some also display dosage control abilities and 2D spatial resolution that are unattainable with bulk transfection techniques. Moreover, they can display single-cell sensing modalities to monitor the membrane permeation process and the cell recovery. An overview of the different technologies discussed here is provided in Fig. 1 and Table 1. For conciseness, we limited our study to in vitro and ex vivo techniques having demonstrated delivery of nucleic acids and proteins, which is the main road toward cell engineering. Except for traditional microinjection, we have here selected the SCTTs having demonstrated almost perfect permeation efficiency and a transfection yield above 50%, which is defined as the fraction of surviving cells that is successfully transfected.

3.1. Microinjection

Microinjection was invented over a century ago [43] and is still widely used to introduce large cargos into cells and sample their content. Simply put, a sharp glass micropipette is punched through a cell's membrane, possibly in a targeted cellular compartment [44], and precise quantities [45] of its content can be delivered (Fig. 2A). Virtually any cargo type can be microinjected. The technique is ideally suited for processing samples of rare and precious cells and delivering very large cargo, such as during in vitro fertilization or nuclear transfer for cloning [14]. Apart from the difficulty of targeting small cells and cells in suspension, and the contamination-prone open dish procedure, microinjection suffers from low throughput and tedious operation: a skilled technician may process only tens of cells per hour.

Subsequent work has focused on automation: thanks to a microfluidic trap array with an open top, up to 250 suspended cells can be held at the same time under a microscope camera. Then, an image analysis routine detects the position of the cells and instructs a robotic arm-mounted pipette to target them for injection [46]. As such, this is an improvement for the processing of small pools of rare cells. However, the throughput is still limited by the holder size, limiting the utility of the technique for most applications. More recently, an innovative microfluidic approach consisting of a 2D array of single-cell traps enclosed in a microfluidic device has been developed. Etched in a silicon substrate, each trap contains a sharp, sub micrometer-sized spike and aspiration perforations [47]: Cells aspired in the traps get punctured by the spike. After their release and incubation with the cargo solution, the large, single pore created on the cell membrane allows for the uptake of large molecules (Fig. 2B). Remarkably low toxicity is shown while achieving more than 75% of plasmid transfection efficiency in primary human T cells, which makes it a promising tool for the delivery of CAR-encoding nucleic acids. However, the current design comprises 2500 capture sites per mm^2, and processing of millions of cells would require large devices possibly incompatible with microfluidics. This invention is currently being developed by a startup, Basilard BioTech, under the name SoloPore.

3.2. Electroporation

In electroporation, electric current pulses are delivered to a cell to transiently permeabilize the plasma membrane. Electroporation was popularized in the early 1980's [48] and is now widely used in

Table 1
Summary of SCTTs.

	Automated Microinjection	Microfluidic Microinjection	Cell Squeeze	Microfluidic Vortex Shedding	In-Flow Electroporation	Nanopore/ Nanostraw Electroporation	Electroporation on Microelectroe Array	Optical injection	Plasmonic Optical Injection
Delivery efficiency for small molecules*	Dextran: 58–88%	PI:93%	Dextran 3 kDa:50–90% 70 kDa:25–45% Antibodies:~35%	Not mentioned	Dextran 10 kDa: up to 95%	PI: >95% miRNA: Up to 100% Protein: Up to 80%	PI: 90%	Calcein efflux: up to 100%	Small Dye: 95% Dextran 10 kDa: 40%–90% 500 kDa: 25%–50% 2000 kDa: 45%
Survival rate	63–82%	~100%	50–95%	70%–95%	60%–90%	90–100%	Not mentioned	60%–90%	70–95%+
pDNA and mRNA transfection efficiency	pDNA: 18% (expression) mRNa: up to 80%	pDNA: 50–90%	(Electrophoresis aided) pDNA: up to 90%	mRNA: Up to 65%	pDNA: 70% - 90%	pDNA: 70%–90%	Not mentioned	pDNA: 55%–90%	pDNA: 45%–100%
Adherent cells	Yes	No	No	No	No	Yes	Yes	Yes	Yes
Suspended cells	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Cell amount / rate	~250 cells per chip ~2 min per chip	10^4 per chip, 2500 / mm^2 of chip surface	10^6–10^7 per chip (clogging) Up to 10^7 cells/s	2 × 10^6 cells / s	10^4–10^6 cells/s	4 × 10^4 per cm2	~100–10^4 per chip	5 cells/s, +10^6 per dish	20 cells/s in single cell mode, +10^6 per dish
Single cell addressability of transfection	Yes	No	No	No	Through IM-triggered EP	No	Yes	Yes	Yes
Built-in monitoring modalities	Microscopy	–	–	–	IM DEP sorting	Cytosol sampling (with nanostraws)	IM DEP	Microscopy	Microscopy
Chip integration	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Main advantages	Delivery of large cargos, targeting subcellular compartments, Dosage control	Efficiently delivers plasmids in primary cells.	Simple to scale up, Little cell disruption, Fast processing rate	Simple to scale up, Little cell disruption, Fast processing rate	Efficient NA delivery, Fast processing rate, Can comprise monitoring modalities	Delivery of very large plasmid possible, Little cytotoxicity, Dosage control	Spatially-resolved delivery, comprises many monitoring modalities.	Spatially-resolved delivery, Virtually sterile.	Spatially-resolved delivery, Highest transfection efficiency.
Main disadvantages	Very slow processing rate. Open dish environment. Low plasmid expression if not injected in nucleus.	Challenging to scale up.	No unaided delivery of plasmids. Cell size variations may alter molecular uptake.	Lower efficiency compared to other in-flow techniques.	Possible disruption of cell function.	Challenging to scale up.	Limited processing rate and scalability.	Slow processing rate. Sensitive to laser misfocusing.	Use of nanoparticles or microfabrication intensive substrates.
Relevant applications	Nuclear transfer, Injection of particles	Car-T, NA delivery	Cancer vaccines, small molecule delivery	Car-T, mRNA delivery	Car-T, NA delivery	iPSC generation, Car-T	Tissue engineering, Clinical trials in a dish	Tissue engineering, Clinical trials in a dish	Tissue engineering, Clinical trials in a dish
Commercial applications / startups	–	Basilard Biotech	SQZ Biotech	Indee Labs	MaxCyte, Kytopen	–	–	–	TrinCE
References	[45,46]	[47]	[19,26,54,56,87–89]	[90]	[61–68,71]	[57,58,72–82]	[59,60,83–85]	[100–103,105]	[55,111,113–122,125,126]

Table 1) Summary of the SCTTs presented in this review. pDNA: Plasmid DNA; PI: Propidium Iodide; IM: Impedance Monitoring; NA: Nucleic Acids; DEP: Dielectrophoresis * The delivery efficiency for small fluorescent dyes is representative of the permeation efficiency. Dextrans are used as model cargo molecule to evaluate a transfection technology's ability to deliver proteins and small macromolecules in cells, compared to large nucleic acids.

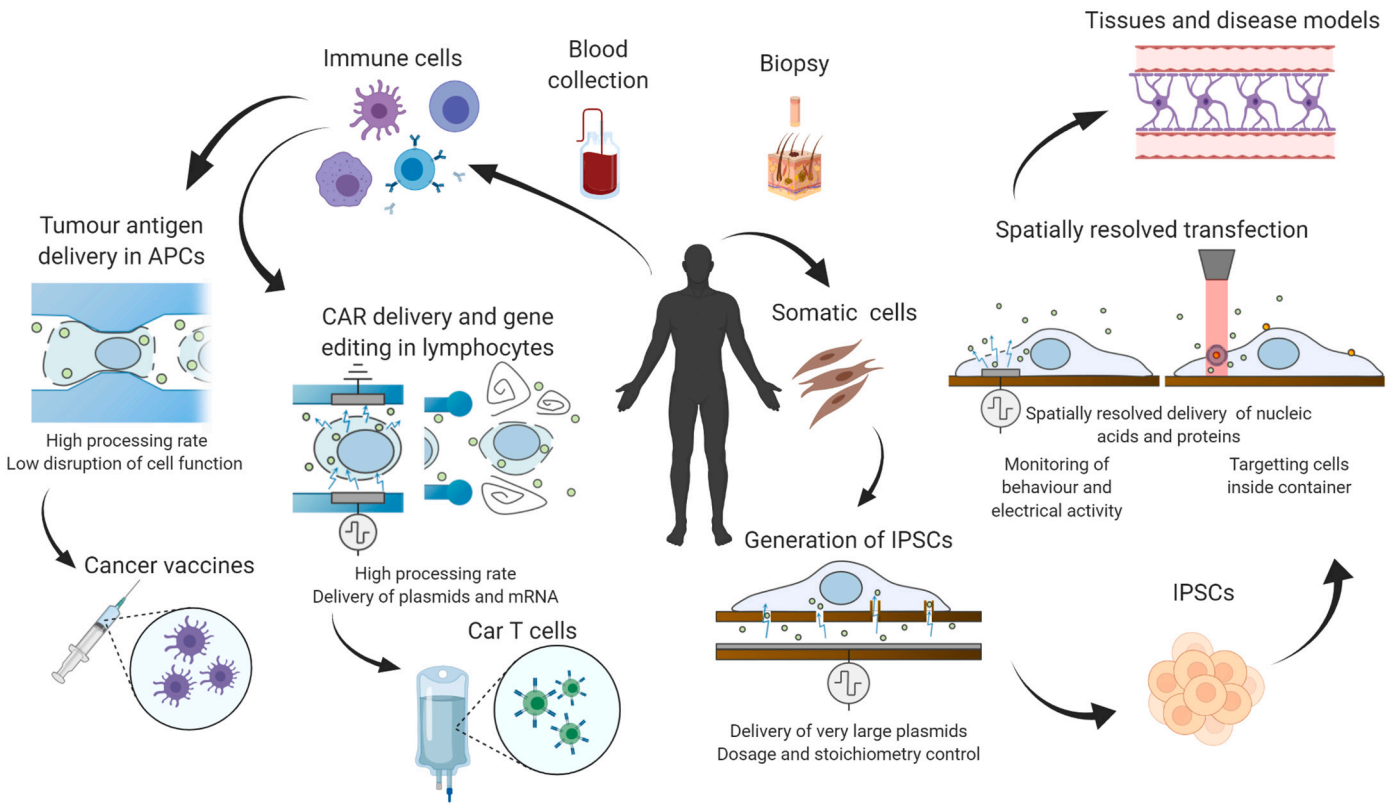

Fig. 1. Main applications of the most mature SCTTs presented in this work. On the left of the figure, immune cells are extracted from blood. In-flow transfection methods with high processing rates are used to deliver tumor antigens or nucleic acids in order to produce cancer immunotherapies. On the right, somatic cells such as skin fibroblast are taken from a tissue biopsy. Delivering large reprogramming plasmids in those cells with nanopore electroporation generates induced pluripotent stem cells. Then, the spatially-resolved delivery of differentiating factors in those iPSCs is used for the creation of more complex disease models and tissues with spatially designed architecture. Created with BioRender.com.

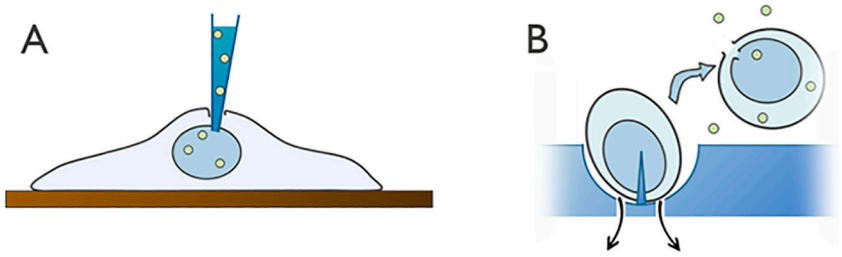

Fig. 2. Microinjection implemented in SCTTs. A) Traditional microinjection B) Microfluidic microinjection.

commercial transfection devices (for instance, the Nucleofector, commercialized by Lonza and the Neon Transfection System, marketed by Thermo Fisher Scientific). GMP-compliant platforms for non-viral transfection mainly rely on electroporation. They can make use of disposable cuvettes (Lonza's 4D-Nucleofector, Cellectis's Pulse Agile), enclosed fluidic systems with disposable elements (MaxCyte's devices), or fully integrated and automated cell manufacturing platform (CliniMACS Prodigy). They typically perform bulk electroporation: suspended cells are mixed with the cargo of interest and dispensed between two large electrode plates fitted in a cuvette. As a high voltage pulse is applied between the two plates, cytotoxicity is caused by over-exposure to high electric fields, pH changes, heating and release of toxic metal ions. The heterogenous distribution of the electric field causes variable exposure at the single-cell level, impacting individual cell survival and transfection efficiency [49].

Electroporation is a valuable technique to deliver nucleic acids: it triggers the uptake and intracellular trafficking of plasmids toward the nucleus, and electrophoresis during electroporation has been shown to drag nucleic acids into the target cells [50–52]. It is a major advantage over other physical transfection methods, which may struggle with the delivery of large charged molecules that are electrostatically repelled by the cell membrane or stall near the entry point in the cytosol [53–56].

Miniaturization of electroporation devices allows to perform controlled permeation at the single-cell level and offers other advantages: 1) Reduction of the distance between the electrodes allows to achieve similar electric fields, thus electroporation, at lower voltages, and smaller electrodes can apply highly localized currents to only electroporate a patch of the cell membrane for reduced cytotoxicity [57,58]. 2) In microdevices, reliable cell positioning with respect to the electrodes, as well as predictable electric fields further improve transfection efficiency [49]. 3) Miniaturization of the electroporation electrodes enables integration in devices possibly comprising thousands or millions of them, i.e. microelectrode arrays, that may also feature additional cell interfacing modalities [59,60]. Thus, single-cell electroporation devices can display higher efficiency, cell survival and control over transfection. Single-cell electroporation devices can be divided in

three main categories: In-flow electroporation, nanopore electroporation and electroporation on microelectrode arrays (Fig. 3).

3.2.1. In-flow electroporation

In-flow electroporation devices are simple microfluidic devices typically made of PDMS, glass or silicon fitted with electrodes. Suspended cells are flown in the microchannel, where an electric potential is applied between the electrodes, creating regions of high electric field strength. The duration and amplitude of the electric "pulse" applied to the cells can be adjusted by modulating the flow rate, channel geometry and number of electrodes. The ability to process cells suspensions grants those devices high throughput, from 10^4 to 10^8 cells per minute with close to 80% efficiency and cell viability [61,62].

Multiple approaches have been taken to increase transfection efficiency, e.g. fitting electrodes at both ends of the microchannel and alternating regions of high and low electric fields to permeate the cells [61,62], or hydrodynamically rotating the cells in the channel to improve efficiency by uniformly exposing the cell membrane to high electric fields [63–65]. A different approach is patterning microelectrodes in the channel. Wei et al. developed a laminar flow electroporation device that uses hydrodynamic flow focusing to align the cells between two longitudinal electrodes patterned on the entire length of the channel. The focusing greatly enhanced cell survival and transfection efficiency by reliably positioning the cells in the center of the channel and preventing exposure to the harsh conditions in the vicinity of the electrodes [66]. Addition of a dielectrophoretic sorter at the end of the electroporation channel to automatically remove cells that were lysed by the electroporation allowed to transfect fragile or hard-to-transfect cells, with viability varying from 20% to more than 80% in certain cases thanks to sorting [67]. Another device, featuring interdigitated comb electrodes that invert the electric field multiple times along the channel, boasts a very high delivery yield [68]. For most cell lines, in-flow electroporation often displays efficiencies in delivery and survival rates in the range of 70%–80%, sometimes up to 90%. Those numbers vary greatly with different cell types and can fall by half for some primary cells, showing that some cell types may be more amenable to electroporation than others.

Fitting electrodes in the microchannel opens the door to label-free electrical measurements [69]. Applied to electroporation, changes in membrane conductivity induced by electroporation can be quantified when a cell passes between a pair of electrodes by impedance measurements [70]. Impedance measurements have also been used to detect cells, trigger electroporation and monitor its outcome [71]. These built-in process monitoring strategies would be a step toward in-line quality control in cell therapy manufacturing.

Overall, in-flow electroporation devices show high processing rates, relatively high delivery efficiency for a variety of molecules and can easily integrate sorting and monitoring modalities based on electrical measurements. That is why they are a promising tool for manufacturing of CAR T therapies by delivering CAR-encoding plasmids or mRNA into T cells as well as gene editing RNP complexes. Consequently, in-flow electroporation emerges as a valuable tool for CAR T and APC engineering, thanks to its ability to deliver nucleic acids and proteins alike. The main commercial instance of in-flow electroporation is currently marketed by MaxCyte, which uses its proprietary, GMP-compliant, in-flow electroporation device to pursue clinical trials based on mRNA-engineered CAR T cells (NCT03608618). Finally, Kytopen, a recently founded biotech startup, is developing an in-flow electroporation technology aimed at gene-editing and research on cell therapy. The technology, called FlowFect, uses a microchannel with a narrow section. There, the electric fields are locally amplified, which allows for a controlled and efficient electroporation of cells flown in the device.

3.2.2. Nanopore electroporation

In nanopore electroporation devices, cells are funneled into nano-sized channels connected to a chamber filled with the cargo solution. Electric currents passing through the nanochannel are strongly condensed and electroporate a localized area of the cell membrane. Maintaining the electric field electrophoretically "injects" charged molecules in amounts controlled by the pulse duration. Nanopore electroporation offers virtually no cytotoxicity and fast expression of the delivered plasmids [72]. This efficient delivery platform was later applied to monitor the up- and downregulation of oncogene networks with delivery of miRNAs and RNA molecular beacons in AML cells [57]. In further nanopore electroporation designs, cells were grown on microfabricated planar nanoporous membranes with 40,000 pores per cm^2. Proof-of-concept studies have shown that the platform can transfect cardiomyocytes and also deliver very large reprogramming plasmids (13 kb) in Mouse Embryonic Fibroblasts (MEF) with 90% transfection efficiency, generating close to 1000 times more iPSC colonies than bulk electroporation [73]. Additionally, successful transfection of mice embryo primary neurons [74], reprogramming of MEF into iPSCs [75] and transdifferentiation of MEFs into neurons was demonstrated. Expression of neuronal phenotypic markers was faster than upon bulk electroporation and viral transfection [58].

Further improvements enabled the attraction of suspended immune cells to the pores by dielectrophoresis, extending the utility of the platform to non-adherent cells. After delivery of a CAR/GFP-reporter plasmid in natural killer cells, around 80% of the cells loaded on the chip showed GFP expression with little cell death [76]. As an alternative to dielectrophoresis, centrifugation of suspended cells deposited on the nanoporous membrane permitted to yield similarly high delivery efficiencies of proteins and nucleic acids and cell survival [77]. Such high transfection yield and low cell disruption combined with the possibility of using cell suspensions for higher throughput make it a very promising example of SCTT for both adoptive immunotherapies and iPSC generation. However, increasing the throughput will likely encounter the same problems as in the microfluidic microinjection device [47]. Prolonging the pores using nano-sized tubes [78–81], called nanostraws, additionally enables sampling of the cytosol thanks to a tight seal between the tube apertures and the cell membrane [82]. Finally, electroporation on nanostraws has been shown to minimally disturb cell division and gene expression [81].

The possibility to deliver controlled doses of very large plasmids with nanopore electroporation is an important step in iPSC research. Not only does packing of all reprogramming TFs in a non-viral vector limit the

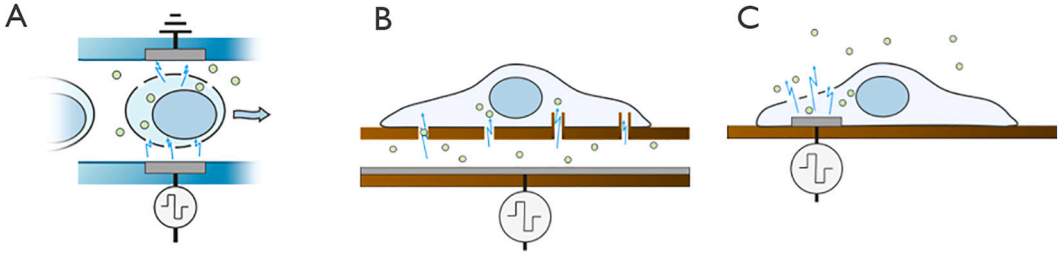

Fig. 3. Electroporation implemented in SCTTs. A) In-flow electroporation B) Nanopore / nanostraw electroporation C) Microelectrode array electroporation.

safety risks associated with viruses and possible uncontrolled expression of oncogenes [30], but tuning the ratios of the TFs including miRNA in a single plasmid to improve reprogramming efficiency will allow researchers to overcome the biological processes currently impeding cell reprogramming [7,58]. The co-delivery of a cocktail of mRNAs and miRNAs in such a device could also offer a higher-efficiency alternative to plasmids [7]. Combining a nanopore electroporation/Nanostraw device with a microfluidic system could in principle allow to formulate a solution composed of different cargos. Different cargo solutions could be sequentially or simultaneously pumped into the system for delivery, which would allow dosage and temporal control over transfection.

3.2.3. Electroporation on microelectrode arrays

The last type of electroporation devices consists of microelectrode arrays (MEA). In a MEA, micrometer-sized electrodes can electrically interface with individual cells for electrical stimulation and monitoring with an enormous versatility and at single-cell resolution. For instance, electrophysiological recordings and impedance monitoring offer a label-free real-time assessment of cell function and status [59]. Impedance monitoring can also be used to detect cells growing on top of the electrodes and to evaluate the quality of the cell-electrode coupling [83]. While there are examples of cell transfection on larger electrodes, instances of single-cell transfection on MEAs are scarce and only a few key studies that demonstrate proof of concept can be found.

By fitting a MEA in a microfluidic chamber comprising an array of electroporation/impedance monitoring electrodes and an array of positioning dielectrophoresis electrodes [60,84,85], suspended cells flown in the device can be "trapped" by dielectrophoresis to promote adherence on the electroporation/impedance monitoring electrodes. An electric pulse can deliver cargo such as plasmids into adherent cells while adhesion dynamics, resealing of the membrane and cell survival are assessed in real time through impedance monitoring.

MEAs are the ideal tool to evaluate drug efficacy and toxicity and study disease models of electrogenic cells. In our research, we are leveraging a state-of-the-art CMOS MEA for single-cell transfection and monitoring. The high density of individually addressable electrodes enables stimulation and electroporation, impedance monitoring and electrophysiological recording with single-cell addressability [59,86]. The chip has been used to successfully deliver plasmids in HeLa cells and human fibroblasts (our unpublished results). As for nanopore electroporation, the small size of the electrodes guarantees minimal cytotoxicity.

The main strength of MEA electroporation, compared to most other SCTTs, is plasmid delivery with single-cell addressability. By sequentially delivering different reprogramming or differentiation plasmids into adjacent cells, it is possible to generate complex cell patterns on the surface of the chip, to "print tissues". Combined with iPSC technology, MEA electroporation has profound implications in the creation of personalized disease models, in which the monitoring abilities can be used for label-free assessment of pathological cell functions and responses to drugs or gene editing treatments [42]. As such, it could greatly benefit the creation of brain-on-a-chip models from patients suffering from neurodegenerative diseases.

3.3. Mechanical stress in microchannels

In 2013, an intracellular delivery method based on rapid mechanical deformation of the cell's membrane rose to prominence. The technique, coined "cell squeezing", is outstandingly simple: as the cell flows at high speed through a narrow microchannel in a silicon chip, the transient pores created during the "squeezing" allow for molecules in the cell's surroundings to diffuse into its cytosol [54] (Fig. 4A). The technique can process up to millions of cells per minute and only requires a controllable pressure source to be operated. The channel constriction's geometry and number, as well as the target cell flow rate need to be adapted to specific cell types and sizes. Disadvantages of the technique may arise in the tedious optimization of the constriction geometry and flow rate for each cell type and the cell-to-cell variability in uptake of molecules due cell size distribution. Finally, it would benefit from the integration of monitoring modalities to track the delivery process.

Cell squeezing has been shown to deliver a large panel of small or uncharged molecules: dextrans, proteins, RNPs, nanoparticles and siRNA could be delivered in varied cell lines and primary cell types [19,26,54,87–89]. Multiple primary cell types were processed and kept above 75% of cell viability, while embryonic stem cells retained above 50% viability. They demonstrate the generation of iPSCs by sequential delivery of the four Yamanaka factors as recombinant proteins in human fibroblasts, resulting in ten times more colonies than when performed in a commercial electroporator [54]. Moreover, cell squeezing, compared to bulk electroporation, does not disrupt cell function: T cells edited via squeezing had higher tumor-killing properties than cells processed with bulk electroporation and displayed no release of cytokines, and squeezed hematopoietic stem cells had undisturbed proliferative and differentiation abilities (bulk electroporated hematopoietic stem cells were heavily disturbed) [19].

Cell squeezing has been developed by a company, SQZ Biotech, investigating the generation of APCs for cancer vaccines and immunomodulation (clinical trial NCT04084951). Protein antigens have been loaded in B cells and successfully presented at their surface. The loaded B cells induced activation and proliferation of CD8+ T cells [26]. In overall, cell squeezing appears as one of the most promising technologies for the development of APCs, with many key advantages. Thanks to a simple and efficient chip design, straight forward parallelization allows for high scalability. Its minimal disruptive effects on cell function are essential in the generation of therapeutically active immune cells.

The delivery of large, charged molecules such as nucleic acids has been rendered possible by the addition of electrodes after the narrow channels. As porated cells flow in between longitudinal electrodes, nucleic acids in solution are introduced by electrophoresis. Squeezing cells followed by electric field-enhanced DNA delivery allowed to lower the electric field strength necessary for transfection. It resulted in almost twice the transfection efficiency, with only a small decrease in survival, compared to cells treated with stronger electric fields without squeezing. This allowed to deliver a GFP plasmid in HeLa cells with efficiency close to 100% and around 90% viability. Plasmids reached the nucleus and was expressed under an hour, due to the disruption of the nuclear membrane by the high electric field generated between the electrodes

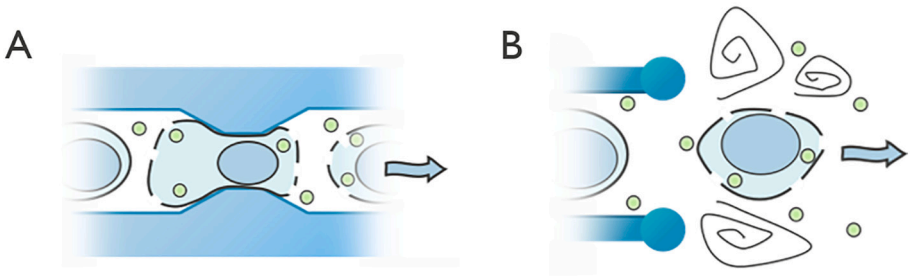

Fig. 4. SCTTs applying a mechanical stress on the cell membrane. A) Microfluidic cell squeezing B) Microfluidic vortex shedding (μVS).

[56]. This electric-field enhanced delivery of nucleic acids could allow for cell transduction with mRNAs for safer CAR delivery.

Other devices based on microfluidic mechanical membrane disruption have been developed. For instance, a technique based on shear stress-induced membrane poration uses vortices created at the end of a microfluidic channel at high flow rates (Fig. 4B). The invention, coined microfluidic vortex shedding (μVS), could deliver a 1 kb GFP mRNA in primary human T cells with more than 60% transfection efficiency and 80% cell viability while minimizing toxic side effects. Moreover, the permeation process does not rely on cell size and might be applicable to any cell type without discrimination. Finally, the device can process millions of cells per second, compatible with the output demanded for the generation of CAR T cell therapies [90]. The company behind μVS, Indee Labs, has built the μVS Delivery System™ around this concept. The device, that uses disposable cartridges and tubing, is aimed at offering an affordable way to deliver nucleic acids, proteins or gene editing constructs to T cells. Other examples of shear stress-induced membrane permeation have also demonstrated intracellular delivery of large molecules, such as DNA nanostructures [91,92]. For instance, in sonoporation, microbubbles exposed to ultrasounds rapidly expand and collapse, generating large shear stresses that permeate the nearby cells. While this technique has not yet reached high efficiency, it has shown close to 50% plasmid transfection efficiency in suspended cells [93], delivery of large dextran molecules in adherent cells [94] and single-cell addressability [95]. For further information, we direct the reader to other works [14,96,97].

3.4. Optical injection

In optical injection (also called photoporation or optoporation), a high-intensity laser pulse is condensed on a sub-micrometer dot, creating a nanosurgery tool able to dissect the plasma membrane and cellular compartments for molecular cargo delivery. The advantages of the technique are compatibility with most microscopy setups, processing of adherent and suspended cells, single-cell addressability and a virtually sterile procedure: cells can be porated without direct exposure to the environment [98]. The technique has been applied to deliver a wide range of molecules, including plasmid DNA, mRNA, siRNA and proteins [14].

The most effective light source for single-cell transfection are femtosecond lasers: ultrashort, high-energy pulses are focused on the membrane and generate a pore as big as a micrometer [99] for efficient plasmid delivery [100] (Fig. 5A). Combination with a microscopy setup and motorized sample stage creates a microinjection-like system, in which an operator manually searches for targets in a cell population and triggers the laser pulse. This system has shown consistently high efficiencies and cell viability in delivering plasmid molecules [98,101–103]. It has also been applied to stain selected retinal cells and study actin structures within whole explanted rat eyes [104]. In principle, the same method could be used for in vivo delivery of plasmids. Automation of the process increases throughput and reduces process variability, for instance through combination with image analysis software and cell selection routines [105]. Optical injection has also been adapted to deliver reprogramming minicircle vectors in human dermal fibroblasts as they flow in a microcapillary [106,107]. While formation of iPSC-like colonies has been shown [107], the 1% transfection efficiency of this method is marginalized by other cell suspension methods, like dielectrophoresis-assisted nanopore electroporation of plasmids or cell squeezing with reprogramming factors in purified protein form.

The main disadvantages of femtosecond-pulse transfection are the critical importance of vertical alignment between the laser and the cell membrane: micrometer-sized mismatches can drastically reduce the poration effect of the laser pulse. Moreover, the high price of the required light sources is an important access barrier [108]. As an alternative, researchers have explored optically absorbent materials (such as ITO and gold) that focus and amplify the energy of weaker lasers while solving the problem of vertical alignment. In those materials, the light is converted into heat, which induces the creation and rapid collapse of microbubbles near the cell membrane, which gets permeated by the fluid shear stress that is generated in the process [109]. Plasmonic materials are either used as a cell culture substrates and surfaces close to the cells for optical injection [110–116,119] (Fig. 5C) or as nanoparticles incubated with the cells for adsorption on their membrane [55,108,117,118,120,121] (Fig. 5B). Plasmonic nanoparticles extend the use of the technique to suspended cells and might have the advantage of simplicity over microfabrication-intensive substrates. Nanoparticle-enhanced optical injection has been shown to have low toxicity on primary T cells [122] and limited negative impact on cell behavior [123]. Antibody-functionalized nanoparticles allow targeting of a specific cell type within a mixed sample, and the clustering of nanoparticles on the targeted cells amplify the poration effect, reaching high plasmid transfection efficiency and cell viability in primary T cells [117]. The method has been applied to optical injection in a capillary. In other work, nuclear poration was achieved with functionalized nanoparticles delivered via electroporation and targeted to the nuclear envelope [124].

Finally, an automated nanoparticle-enhanced optical injection has been developed, based on a motorized sample stage and image recognition software. This Spatially-resolved Nanoparticle-enhanced Photoporation, coined SNAP, can process up to 200 cells per second. It can deliver large macromolecules in cells to either create predefined patterns of laser exposure or recognize individual cells based on certain characteristics, like a fluorescent label, and expose them to a porating laser pulse [55]. SNAP is the core technology behind LumiPore, a product currently developed by the startup TrinCE. TrinCE views tissue engineering and CAR-T cells as the main applications for their technology. For a convenient and reagent-free harvesting of cells transfected on a plasmonic surface, a surface coating with temperature-dependent cell adhesion properties has been developed [125]. Such strategies would allow for entirely contactless and automated cell transfection and harvesting. They also combined cationic polymers to enhance the delivery of plasmid DNA [126], which is an interesting combination of delivery methods to enhance transfection efficiency. It was developed as a platform to efficiently process hard-to-transfect cells, and boasts more than 80% of transfection efficiency in primary endothelial cells and MEF, with ~80% and ~ 100% of viability for those cell types, respectively. Interestingly, the different cationic polymers tested did not have the same toxic effects on both cell types.

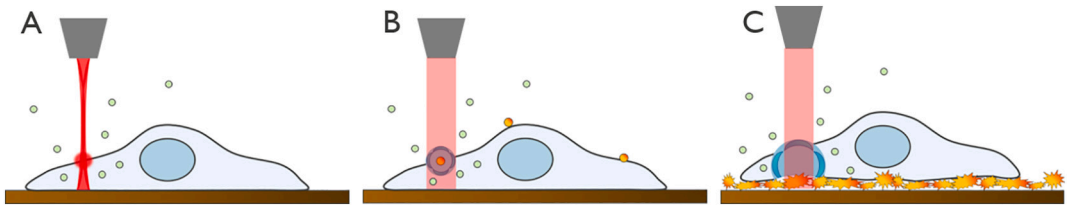

Fig. 5. Different implementations of Optical Injection A) "traditional" Optical Injection, such as fs-laser optical injection B) Plasmonic nanoparticle-based optical injection C) Plasmonic surface-based optical injection.

4. Toward clinically relevant, GMP-compliant SCTTs

Medicinal products that have been granted marketing authorization have to comply with GMP as early as from the clinical investigational phase. That is why GMP compliance should be considered as early as possible when developing a transfection technology with intended clinical applications. As our goal is to stimulate the translation of promising technologies to the clinics, we would like to put emphasis on how GMP compliance can be integrated in the design of SCTTs. Currently, there are only few GMP-compliant SCTTs commercially available. Electroporation platforms are the most predominant, such as in MaxCyte's in-flow electroporation devices.

GMP are a set of rules and guidelines overseeing the manufacturing of pharmaceutical products and ensure that clearly defined quality attributes are met. GMP focus on managing the risks involved in each manufacturing step by thoroughly documenting, validating and controlling processes. GMP are linked to an entire manufacturing process: they guide the manufacturing of medical products from starting material to stored, finished product in its final packaging, ready to be shipped to the patients. As such, GMP also cover topics like receiving goods, maintaining and cleaning the manufacturing premises, controlling suppliers, training employees and labelling of reagents and products. Box 1 describes the main GMP requirements for a generic process for manufacturing advanced therapy medicinal products [127]. When considering transfection as part of a cell product manufacturing, we can translate the points listed in Box 1 into features that must be implemented in a GMP-compliant SCTT.

1. The transfection must be reliable over time and generate a uniform cell population. Thus, an SCTT must display high transfection efficiency and cell survival. Uniformity is achievable with 1) precise dosage-controlled intracellular delivery; and 2) by limiting disruptive side effects. Dosage control has been demonstrated in microinjection and nanopore electroporation. On the other hand, nanoparticle-enhanced optical injection, where a variable number of nanoparticles may bind cells, or cell squeezing, which would not perform uniformly on cell samples with varying sizes, might struggle in delivering a uniform amount of cargo. Cell squeezing [19], nanopore electroporation [81] and some forms of optical injection [122,123] have been shown to minimize toxic side effects. The extensive approaches used by DiTommaso and colleagues [19] or Tay and Melosh [81], based-on functional and gene expression analysis are a solid foundation on which to build a standard analysis.
2. Real-time and in-process quality control strategies are necessary to track the transfection process efficiency and observe early on any deviation from quality requirements. Examples of built-in transfection monitoring are found in electroporation devices, where processed cells can be counted, and membrane permeation can be verified by impedance monitoring. Dielectrophoresis sorting of viable cell is also an elegant way to ensure product quality. In principle, these monitoring strategies could be, and should be, incorporated into other SCTTs that are not based on electroporation.
3. The transfection must be sterile and safe, and output a cell product free of contaminant like microorganisms or chemicals. SCTTs enclosed in microfluidic devices, and OP can conveniently maintain the sterility of the processed cells. Contamination risks generated by open dish techniques, like microinjection, need to be managed by performing the technique in an isolator or cleanroom, which pose additional challenges related to process and equipment validation. Moreover, contact between cells and non-disposable material should be minimized, thus techniques based on disposable devices, such as microfluidic chips and tubing, are best suited. For reusable devices on the other hand, such as MEAs too costly to dispose of, validated cleaning procedures must be put in place. Only GMP-grade reagents and biocompatible materials should be used, and their availability should be kept into account.
4. Automation of the process is necessary to reduce variability and possible mistakes leading to product contamination. Points to consider are the automation of the transfection itself, as well as the operation of the entire set up. Questions to keep in mind are: Are the fluidics control, the stimulus application and the cargo formulation automatable? In optoporation, is the cell selection and sample movement automated? As a general principle, SCTTs processing cell suspensions benefit from a simpler operation and automation than devices handling adherent cells, which need to be cultured on the device.
5. Commercially viable product requires a scalable manufacturing process. SCTTs with throughput high enough for their applications are necessary. In flow methods, such as cell squeezing and in-flow electroporation can process millions of cells in minutes and can be parallelized and combined with automated microfluidic handling to produce large quantities of cell product. Device cost is also to consider: MEA chips and powerful optoporation lasers might prevent their parallelization.
6. Process validation is the documented demonstration that a process performs reliably as intended and is key to GMP compliance. Validation shows that the process is adequate for its intended operation and that it meets all predefined requirements. Thus, each possible usage situation that could critically affect the product and process quality must be validated. While every SCTT can in principle be validated, the complexity of the method usually determines the complexity of the validation process, which should be taken into consideration during the design phase of the SCTT. electroporation for instance, uses a large pulse parameter space with complex interactions that are hardly predictable. A device that allowed complete freedom over the electroporation parameters could not realistically be validated, as each combination of the parameter space would have to be thoroughly tested. A viable approach is to limit the user's choice to a few sets of parameters, validated for certain cell types or cargos. It is the approach used by Lonza and their Nucleofector device. Moreover, any software control used during the process needs to perform in any situation encountered by the user: any automation must be proven to be reliable. In image-guided optoporation, the cell recognition routines need to be proven effective in every situation possibly encountered during use, such as different cell morphologies, surface densities, etc.

5. Conclusion/outlook

Cell therapies have demonstrated irrefutable success in clinical settings, and the next challenges are in scaling up the manufacturing of cell products. The SCTTs presented above offer such opportunity in multiple ways. First, these transfection methods can eliminate the tremendous costs, manufacturing complexity, regulatory burden and safety concerns linked with viral vectors. Second, their higher efficiency, propensity for automation enablement, versatility with cell types and cargos and built-in process monitoring modalities guarantee convenient integration in industrial setups, where they ensure the manufacturing of high-quality cell products. Finally, some unique features, such as dosage control, minimal cell perturbation and single-cell addressability among a population will uniquely enable applications previously unthinkable with bulk transfection methods.

Still, much remains to be done to mature the SCTTs presented in this review. Besides optimization of the membrane disruption stimulus, many aspects of transfection are relatively unexplored. Improving the efficacy of SCTTs by combining some of their features, or improving transfection efficiency by using chemical carriers, could be more common. For instance, combining sonoporation [128] or cell squeezing [56] with electrophoresis-mediated nucleic acid delivery is a great way to enable efficient transfection. The use of cationic polymers to facilitate nucleic acid entry in the cell, rather than naked nucleic acid, could benefit all SCTTs based on mechanical membrane disruption such as cell

Box 1
GMP and QBD.

GMP integrate the Quality By Design (QBD) principles. QBD is a validation strategy dictating that quality is built in the product by understanding the manufacturing process and the risks associated rather than solely screening it at the end. The QBD approach uses statistical, analytical and risk-management methodology (i.e. determination of critical quality attributes and critical process parameters) in the design, development and manufacturing of medical products. One of the goals of quality by design is to ensure that all sources of variability affecting a process are identified, explained and managed by appropriate measures. This enables the finished medicine to consistently meet its predefined characteristics from the start. Here are the key points to consider when developing an SCTT as part of a GMP-compliant cell manufacturing process.

Uniformity / Reproducibility

The process must be stable and reliable and have a predictable outcome. Process variation should be avoided and eliminated if possible.

- Can the outcome of the process be predicted with enough confidence?
- What are the expected transfection success rate or cell viability?
- Is the process stable over time? Are process variations limited with this technique?
- Are there Critical Process Parameters (CPP) that directly influence the Critical Quality Attributes (CQA)? How easy is it to control these with the technique? (i.e., flow rate in cell squeezing, voltage in electroporation, etc.)
- Can you determine the quantity of the starting materials and finished product?

Validation approach

The process needs to be validated. Process validation is the analysis of data gathered throughout the design and manufacturing of a product in order to confirm that the process can reliably output products of a determined standard.

- How complex is the validation of the process? Is there equipment involved and what is its complexity (e.g. pipette versus robot)? Does this equipment need validation too?
- What parameters are critical and in which playing field is the process or technique stable (see also uniformity / reproducibility)?
- Is there a software involved in the detection and automated systems? Does it require validation, or is it built on an approved software?

Use of GMP materials

The materials that are used during the process should not negatively interfere with the cell product. Biocompatibility and animal product-free reagents are key.

- Are disposables used and do they come in direct product contact?
- What about sterility and biocompatibility? Can the materials be used in combination with living cells without affecting their quality?
- What is their extractable / leachable profile? Extractables and leachable compounds (E/L) studies are critical to the identification and quantification of harmful leachable impurities which could migrate from container closure systems, process equipment and packaging to contaminate cell products.
- The used materials should themselves comply to "GMP-grade production". Is it possible to obtain these? Are their suppliers certified for this purpose?

In process controls / monitoring of the process

Built-in monitoring strategies are required to confirm that the process is running as validated and to assess the quality during the process itself.

- Does the technique offer possibilities for measurements during the process? Is it possible to integrate process controls? Are the CPP measurable?
- Is real time monitoring possible? Real time monitoring gives a quicker analysis of the process than microscope or lab testing.
- Can those methods / in-process controls be validated?

Quality control

Quality controls of the outcome of the process on the intermediate or final cell product (transfected cell) need to be performed.

- Is it possible to perform quality controls?
- Can those methods / in-process controls be validated?
- Which quality controls need to be performed?
- How is sampling performed?
- Can destructive sampling be avoided?

Cleanability

For non-disposable, reusable direct product contact equipment, it is necessary to prevent cross contamination or other contamination by cleaning / sterilizing the equipment.

- Is the used equipment cleanable?

Prevention of microbial or cross contamination / Sterility / Safety

Final products should be free from microbial contamination and endotoxins, absence of mycoplasma, in vitro adventitious agents and replication-competent retrovirus.

- Can direct product contact with the equipment be reduced?
- Is disposable / single use equipment involved?
- Is it possible to reduce the risk of contamination regarding equipment, materials, human intervention, and other agents?
- Is it possible to perform the process in a closed environment/under laminar flow/in an isolator?
- Is it possible to integrate the technique in a chip integration or closed dish environment?

Scalability in general

- Is the technique able to produce transfected cells on a large scale in a shorter timeframe?
- Are the transfection efficiency and cell viability high enough to generate the needed amount of cell product without extensive sorting?

Automation possible?

Can the process be automated? Automated detection, control systems, execution?

- Are manual interventions needed? Can the process be automated (in a cost-effective manner)?
- Are there detection methods or software that can assist in automation of the process?
- Is maintenance and calibration possible and easy for the automated systems?

Cell collection / harvesting

- Is the harvesting technique a time-consuming process?
- Is the cell viability not impacted?
- Are there additional contamination risks upon harvesting of the transfected cells?

squeezing and optical injection [126]. Another point to consider is the necessary distinction between the concepts of intracellular delivery and the actual biological effects generated by the delivered molecules. Optimization of the cargos' biological activity, such as in [18], and increasing our understanding of the triggered cellular processes, like in [58], is still required. Characterization of the disruptive effects that permeation has on cell function is another key to improve the efficacy of cell therapies and the relevance of disease models in a dish. As SCTTs are the most relevant to clinical applications when they have demonstrated efficacy on primary cells, each technology's effect on the viability and function of primary cells should be systematically studied.

Importantly, more applications should explore the use of SCTTs and drive their development. For instance, the field of tissue engineering and organs-on-a-chip would certainly benefit from techniques like optical injection, that could in principle transfect and differentiate cells to create complex cellular patterns. The fact that this has not been reported yet shows the effort that remains to be done in applying the unique abilities of SCTTs to applications that could benefit or be enabled by them. On the other hand, the very specific needs of certain applications should also be driving the development of dedicated, specialized SCTTs. It should be clear that no single SCTT can cater for all cell-based medicine applications discussed in this paper. Rather, developers of clinical applications should try to match their needs with the most suitable transfection technology. We hope our work will contribute to this goal.

Finally, a big attention point is compliance with regulatory and quality requirements. Researchers developing clinically relevant SCTTs should consider GMP compliance as early as possible in their designs. The optimization of specific aspects, such as implementing a sterile and reliable process may appear obvious. However, some non-trivial GMP aspects, such as the validation step and its complexity, or the availability of GMP compliant reagents to operate the SCTT, should not be overlooked. Addressing GMP compliance early in the development of SCTTs is key to improve novel technology uptake in clinical applications.

Declaration of Competing Interest

The authors Bastien Duckert, Dries Braeken and Maarten Fauvart declare no conflict of interest.

The author Steven Vinkx declares being employed as QA Project Manager at QbD, 2610 Antwerp, Belgium. QbD offers expertise and solutions in the field of Quality Management, Support and Regulatory Affairs for companies active in Pharma, Biotech, Healthcare and Medical Devices.

Acknowledgements

We thank Michiel Hertegonne for the discussions on Good Manufacturing Practices and Tim Stakenborg for critically reviewing the manuscript. Funding: Bastien Duckert is SB PhD fellow at FWO with grant 1S84919N.

References

[1] S.R. Bailey, M.V. Maus, Gene editing for immune cell therapies, Nat. Biotechnol. 37 (2019).
[2] M.A. Fischbach, J.A. Bluestone, W.A. Lim, Cell-based therapeutics: the next pillar of medicine, Sci. Transl. Med. 5 (2013).
[3] J.N. Brudno, J.N. Kochenderfer, Chimeric antigen receptor T-cell therapies for lymphoma, Nat. Rev. Clin. Oncol. 15 (2018) 31–46.
[4] U. Martin, Therapeutic application of pluripotent stem cells: challenges and risks, Front. Med. 4 (2017).
[5] T.R. Heathman, et al., The translation of cell-based therapies: clinical landscape and manufacturing challenges, Regen. Med. 10 (2015) 49–64.
[6] J. Xin Yu, V.M. Hubbard-Lucey, J. Tang, The global pipeline of cell therapies for cancer, Nat. Rev. Drug Discov. 18 (18–19) (2019).
[7] A.E. Omole, A.O.J. Fakoya, Ten years of progress and promise of induced pluripotent stem cells: historical origins, characteristics, mechanisms, limitations, and potential applications, PeerJ 2018 (2018) 1–47.
[8] D.G. Strauss, K. Blinova, Clinical trials in a dish, Trends Pharmacol. Sci. 38 (2017) 4–7.
[9] R.K. Iyer, P.A. Bowles, H. Kim, A. Dulgar-Tulloch, Industrializing autologous adoptive immunotherapies: manufacturing advances and challenges, Front. Med. 5 (1–9) (2018).
[10] M.P. Stewart, et al., In vitro and ex vivo strategies for intracellular delivery, Nature 538 (2016) 183–192.
[11] https://www.fda.gov/media/108377/download, accessed the 05.11.2020.
[12] M. O'Reilly, J. Dias Alves Pinto, K. Vispute, M. Lowdell, Manufacturing chimeric antigen receptor T cells: issues and challenges, Cytotherapy 21 (2019) 327–340.
[13] X. Wang, I. Rivière, Clinical manufacturing of CAR T cells: foundation of a promising therapy, Mol. Ther. - Oncolytics 3 (16015) (2016).
[14] M.P. Stewart, R. Langer, K.F. Jensen, Intracellular delivery by membrane disruption: mechanisms, strategies, and concepts, Chem. Rev. 118 (2018) 7409–7531.
[15] J.B. Foster, D.M. Barrett, K. Karikó, The emerging role of in vitro-transcribed mRNA in adoptive T cell immunotherapy, Mol. Ther. 27 (2019) 747–756.
[16] C.F. Hung, et al., Development of anti-human Mesothelin-targeted chimeric antigen receptor messenger RNA-transfected peripheral blood lymphocytes for ovarian Cancer therapy, Hum. Gene Ther. 29 (2018) 614–625.
[17] S. Ghassemi, et al., Reducing ex vivo culture improves the antileukemic activity of chimeric antigen receptor (CAR) T cells, Cancer Immunol. Res. (2018), https://doi.org/10.1158/2326-6066.CIR-17-0405.
[18] J.B. Foster, et al., Purification of mRNA encoding chimeric antigen receptor is critical for generation of a robust T-cell response, Hum. Gene Ther. 30 (2018) 168–178.
[19] T. DiTommaso, et al., Cell engineering with microfluidic squeezing preserves functionality of primary immune cells in vivo, Proc. Natl. Acad. Sci. 115 (2018) E10907–E10914.
[20] A.D. Garg, P.G. Coulie, B.J. Van den Eynde, P. Agostinis, Integrating next-generation dendritic cell vaccines into the current Cancer immunotherapy landscape, Trends Immunol. 38 (2017) 577–593.
[21] E. Gilboa, J. Vieweg, Cancer immunotherapy with mRNA-transfected dendritic cells, Immunol. Rev. (2004), https://doi.org/10.1111/j.0105-2896.2004.00139.x.
[22] J.M. Weiss, et al., Efficient responses in a murine renal tumor model by electroloading dendritic cells with whole-tumor lysate, J. Immunother. (2005), https://doi.org/10.1097/01.cji.0000179437.95335.23.
[23] D. Benteyn, C. Heirman, A. Bonehill, K. Thielemans, K. Breckpot, mRNA-based dendritic cell vaccines, Expert Rev. Vaccines 14 (2014) 161–176.
[24] R.L. Sabado, S. Balan, N. Bhardwaj, Dendritic cell-based immunotherapy, Cell Res. 27 (2017) 74–95.
[25] K.A. Hlavaty, et al., Abstract 3187: Engineering a new generation of cell therapies for solid tumor oncology using the SQZ platform, 2019, https://doi.org/10.1158/1538-7445.sabcs18-3187.
[26] G.L. Szeto, et al., Microfluidic squeezing for intracellular antigen loading in polyclonal B-cells as cellular vaccines, Sci. Rep. 5 (2015) 1–13.
[27] H.Y. Wu, C.Y. Cao, The application of CRISPR-Cas9 genome editing tool in cancer immunotherapy, Brief. Funct. Genomics 18 (2019) 129–132.
[28] H. Dolatshad, et al., Application of induced pluripotent stem cell technology for the investigation of hematological disorders, Adv. Biol. Regul. 71 (2019) 19–33.
[29] H. Yin, K.J. Kauffman, D.G. Anderson, Delivery technologies for genome editing, Nat. Rev. Drug Discov. 16 (2017) 387–399.
[30] K. Takahashi, et al., Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.
[31] O. Tsuji, et al., Concise review: laying the groundwork for a first-in-human study of an induced pluripotent stem cell-based intervention for spinal cord injury, Stem Cells 37 (2019) 6–13.
[32] T. Kikuchi, et al., Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model, Nature 548 (2017) 592–596.
[33] L. Cao, L. Tan, T. Jiang, X.C. Zhu, J.T. Yu, Induced pluripotent stem cells for disease Modeling and drug discovery in neurodegenerative diseases, Mol. Neurobiol. 52 (2015) 244–255.
[34] Y. Shi, H. Inoue, J.C. Wu, S. Yamanaka, Induced pluripotent stem cell technology: a decade of progress, Nat. Rev. Drug Discov. 16 (2017) 115–130.
[35] U. Tiemann, et al., Optimal reprogramming factor stoichiometry increases colony numbers and affects molecular characteristics of murine induced pluripotent stem cells, Cytom. Part A **79 A** (2011) 426–435.
[36] E.P. Papapetrou, et al., Stoichiometric and temporal requirements of Oct4, Sox2, Klf4, and c-Myc expression for efficient human iPSC induction and differentiation, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 12759–12764.
[37] S. Yamaguchi, K. Hirano, S. Nagata, T. Tada, Sox2 expression effects on direct reprogramming efficiency as determined by alternative somatic cell fate, Stem Cell Res. (2011), https://doi.org/10.1016/j.scr.2010.09.004.
[38] L. Rohani, et al., Generation of human induced pluripotent stem cells using non-synthetic mRNA, Stem Cell Res. 16 (2016) 662–672.
[39] D. Kim, et al., Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins, Cell Stem Cell (2009), https://doi.org/10.1016/j.stem.2009.05.005.
[40] A. Nityanandam, K.K. Baldwin, Advances in reprogramming-based study of neurologic disorders, Stem Cells Dev. 24 (2015) 1265–1283.
[41] M. Mastrangeli, Building blocks for a European organ-on-Chip roadmap, ALTEX (2019), https://doi.org/10.14573/altex.1905221.
[42] B.J. Wainger, et al., Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons, Cell Rep. 7 (2014) 1–11.
[43] M.A. Barber, A technic for the inoculation of bacteria and other substances into living cells, J. Infect. Dis. (1911), https://doi.org/10.1093/infdis/8.3.348.

[44] J.J. Ludtke, M.G. Sebestyén, J.A. Wolff, The effect of cell division on the cellular dynamics of microinjected DNA and dextran, Mol. Ther. 5 (2002) 579–588.
[45] Y.T. Chow, et al., Single cell transfection through precise microinjection with quantitatively controlled injection volumes, Sci. Rep. 6 (2016) 1–9.
[46] Y.T. Chow, et al., A high-throughput automated microinjection system for human cells with small size, IEEE/ASME Trans. Mechatronics 21 (2016) 838–850.
[47] H.G. Dixit, et al., Massively-parallelized, deterministic Mechanoporation for intracellular delivery, Nano Lett. (2019), https://doi.org/10.1021/acs.nanolett.9b03175.
[48] E. Neumann, M. Schaefer-Ridder, Y. Wang, P.H. Hofschneider, Gene transfer into mouse lyoma cells by electroporation in high electric fields, EMBO J. (1982), https://doi.org/10.1002/j.1460-2075.1982.tb01257.x.
[49] L. Chang, et al., Micro−/nanoscale electroporation, Lab Chip 16 (2016) 4047–4062.
[50] M. Pavlin, M. Kandušer, New insights into the mechanisms of gene Electrotransfer - experimental and theoretical analysis, Sci. Rep. 5 (2015) 1–11.
[51] M.M. Sadik, et al., Scaling relationship and optimization of double-pulse electroporation, Biophys. J. 106 (2014) 801–812.
[52] M.M. Sadik, J. Li, J.W. Shan, D.I. Shreiber, H. Lin, Quantification of propidium iodide delivery using millisecond electric pulses: experiments, Biochim. Biophys. Acta Biomembr. 1828 (2013) 1322–1328.
[53] D. Lechardeur, A.S. Verkman, G.L. Lukacs, Intracellular routing of plasmid DNA during non-viral gene transfer, Adv. Drug Deliv. Rev. 57 (2005) 755–767.
[54] A. Sharei, et al., A vector-free microfluidic platform for intracellular delivery, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 2082–2087.
[55] R. Xiong, et al., Fast spatial-selective delivery into live cells, J. Control. Release 266 (2017) 198–204.
[56] X. Ding, et al., High-throughput nuclear delivery and rapid expression of DNA via mechanical and electrical cell-membrane disruption, Nat. Biomed. Eng. 1 (2017) 0039.
[57] X. Zhao, et al., Nanochannel electroporation as a platform for living cell interrogation in acute myeloid Leukemia, Adv. Sci. (2015), https://doi.org/10.1002/advs.201500111.
[58] D. Gallego-Perez, et al., Deterministic transfection drives efficient nonviral reprogramming and uncovers reprogramming barriers, Nanomedicine Nanotechnology, Biol. Med. 12 (2016) 399–409.
[59] C.M. Lopez, et al., A multimodal CMOS MEA for high-throughput intracellular action potential measurements and impedance spectroscopy in drug-screening applications, IEEE J. Solid State Circuits 53 (2018) 3076–3086.
[60] X. Guo, R. Zhu, Controllable in-situ cell electroporation with cell positioning and impedance monitoring using micro electrode array, Sci. Rep. 6 (2016) 1–8.
[61] N. Bhattacharjee, L.F. Horowitz, A. Folch, Continuous-flow multi-pulse electroporation at low DC voltages by microfluidic flipping of the voltage space topology, Appl. Phys. Lett. 109 (1–5) (2016).
[62] T. Geng, et al., Flow-through electroporation based on constant voltage for large-volume transfection of cells, J. Control. Release 144 (2010) 91–100.
[63] T. Geng, Y. Zhan, J. Wang, C. Lu, Transfection of cells using flow-through electroporation based on constant voltage, Nat. Protoc. 6 (2011) 1192–1208.
[64] J. Wang, Y. Zhan, V.M. Ugaz, C. Lu, Vortex-assisted DNA delivery, Lab Chip 10 (2010) 2057–2061.
[65] M. Zheng, J.W. Shan, H. Lin, D.I. Shreiber, J.D. Zahn, Hydrodynamically controlled cell rotation in an electroporation microchip to circumferentially deliver molecules into single cells, Microfluid. Nanofluidics 20 (2016) 1–12.
[66] Z. Wei, et al., A laminar flow electroporation system for efficient DNA and siRNA delivery, Anal. Chem. 83 (2011) 5881–5887.
[67] Z. Wei, et al., Flow-through cell electroporation microchip integrating dielectrophoretic viable cell sorting, Anal. Chem. 86 (2014) 10215–10222.
[68] A. Adamo, A. Arione, A. Sharei, K.F. Jensen, Flow-through comb electroporation device for delivery of macromolecules, Anal. Chem. 85 (2013) 1637–1641.
[69] J. Chen, et al., Microfluidic impedance flow cytometry enabling high-throughput single-cell electrical property characterization, Int. J. Mol. Sci. 16 (2015) 9804–9830.
[70] S.C. Bürgel, C. Escobedo, N. Haandbæk, A. Hierlemann, On-chip electroporation and impedance spectroscopy of single-cells, Sensors Actuators B Chem. 210 (2015) 82–90.
[71] M. Zheng, et al., Continuous-flow, electrically-triggered, single cell-level electroporation, TECHNOLOGY 05 (2017) 31–41.
[72] P.E. Boukany, et al., Nanochannel electroporation delivers precise amounts of biomolecules into living cells, Nat. Nanotechnol. 6 (2011) 747–754.
[73] L. Chang, et al., 3D nanochannel electroporation for high-throughput cell transfection with high uniformity and dosage control, Nanoscale 8 (2016) 243–252.
[74] D. Alzate-Correa, W. Lawrence, N. Higuita-Castro, D. Gallego-Perez, Isolation and Nanoscale electroporation of primary neuronal cultures, In Situ (2020), https://doi.org/10.1007/978-1-4939-9740-4_15.
[75] L. Chang, C. Chitrakar, M. Nouri, 3D Nanochannel Electroporation for Macromolecular Nucleotide Delivery, 2020, https://doi.org/10.1007/978-1-4939-9740-4_7.
[76] L. Chang, et al., Dielectrophoresis-assisted 3D nanoelectroporation for non-viral cell transfection in adoptive immunotherapy, Lab Chip 15 (2015) 3147–3153.
[77] Y. Cao, et al., Nontoxic nanopore electroporation for effective intracellular delivery of biological macromolecules, Proc. Natl. Acad. Sci. U. S. A. (2019), https://doi.org/10.1073/pnas.1818553116.
[78] X. Xie, et al., Nanostraw-electroporation system for highly efficient intracellular delivery and transfection, ACS Nano 7 (2013) 4351–4358.
[79] V. Caprettini, et al., Soft electroporation for delivering molecules into tightly adherent mammalian cells through 3D hollow nanoelectrodes, Sci. Rep. (2017), https://doi.org/10.1038/s41598-017-08886-y.
[80] Y. Cao, et al., Universal intracellular biomolecule delivery with precise dosage control, Sci. Adv. (2018) 4.
[81] A. Tay, N. Melosh, Transfection with nanostructure electro-injection is minimally Perturbative, Adv. Ther. 1900133 (2019) 1900133.
[82] Y. Cao, et al., Nondestructive nanostraw intracellular sampling for longitudinal cell monitoring, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E1866–E1874.
[83] G. Cellere, et al., A purely electronic method to measure transfection efficiency in a single-cell electroporation biochip, ECS Trans. 6 (2007) 1–11.
[84] X. Guo, R. Zhu, X. Zong, A microchip integrating cell array positioning with in situ single-cell impedance measurement, Analyst 140 (2015) 6571–6578.
[85] T. Zheng, Z. Zhang, R. Zhu, Flexible trapping and manipulation of single cells on a chip by modulating phases and amplitudes of electrical signals applied onto microelectrodes, Anal. Chem. 91 (2019) 4479–4487.
[86] B. Miccoli, et al., High-density electrical recording and impedance imaging with a multi-modal CMOS multi-electrode array chip, Front. Neurosci. 13 (1–14) (2019).
[87] A. Kollmannsperger, et al., Live-cell protein labelling with nanometre precision by cell squeezing, Nat. Commun. 7 (2016) 1–7.
[88] M.T. Saung, et al., A size-selective intracellular delivery platform, Small 12 (2016) 5873–5881.
[89] A. Sharei, et al., Ex vivo cytosolic delivery of functional macromolecules to immune cells, PLoS One 10 (2015) 1–12.
[90] J.A. Jarrell, et al., Intracellular delivery of mRNA to human primary T cells with microfluidic vortex shedding, Sci. Rep. 9 (2019) 1–11.
[91] Y. Deng, et al., Intracellular delivery of Nanomaterials via an inertial microfluidic cell Hydroporator, Nano Lett. 18 (2018) 2705–2710.
[92] M.E. Kizer, et al., Hydroporator: a hydrodynamic cell membrane perforator for high-throughput vector-free nanomaterial intracellular delivery and DNA origami biostability evaluation, Lab Chip 19 (2019) 1747–1754.
[93] Y. Qiu, et al., The correlation between acoustic cavitation and sonoporation involved in ultrasound-mediated DNA transfection with polyethylenimine (PEI) in vitro, J. Control. Release 145 (2010) 40–48.
[94] I. De Cock, et al., Ultrasound and microbubble mediated drug delivery: acoustic pressure as determinant for uptake via membrane pores or endocytosis, J. Control. Release (2015), https://doi.org/10.1016/j.jconrel.2014.10.031.
[95] Z. Fan, H. Liu, M. Mayer, C.X. Deng, Spatiotemporally controlled single cell sonoporation, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 16486–16491.
[96] S. Roovers, et al., The role of ultrasound-driven microbubble dynamics in drug delivery: from microbubble fundamentals to clinical translation, Langmuir 35 (2019) 10173–10191.
[97] A. Delalande, S. Kotopoulis, M. Postema, P. Midoux, C. Pichon, Sonoporation: mechanistic insights and ongoing challenges for gene transfer, Gene (2013), https://doi.org/10.1016/j.gene.2013.03.095.
[98] D.J. Stevenson, F. Gunn-Moore, P. Campbell, K. Dholakia, Single cell optical transfection, J. R. Soc. Interface 7 (2010) 863–871.
[99] H. Niioka, N.I. Smith, K. Fujita, Y. Inouye, S. Kawata, Femtosecond laser nano-ablation in fixed and non-fixed cultured cells, Opt. Express 16 (2008) 14476.
[100] A.A. Davis, M.J. Farrar, N. Nishimura, M.M. Jin, C.B. Schaffer, Optoporation and genetic manipulation of cells using femtosecond laser pulses, Biophys. J. 105 (2013) 862–871.
[101] M. Antkowiak, M.L. Torres-Mapa, D.J. Stevenson, K. Dholakia, F.J. Gunn-Moore, Femtosecond optical transfection of individual mammalian cells, Nat. Protoc. 8 (2013) 1216–1233.
[102] A. Uchugonova, K. König, R. Bueckle, A. Isemann, G. Tempea, Targeted transfection of stem cells with sub-20 femtosecond laser pulses, Opt. Express 16 (2008) 9357.
[103] H.G. Breunig, A. Batista, A. Uchugonova, K. König, Cell optoporation with a sub-15 fs and a 250-fs laser, J. Biomed. Opt. 21 (2016), 060501.
[104] S. Batabyal, Y.-T. Kim, S. Mohanty, Ultrafast laser-assisted spatially targeted optoporation into cortical axons and retinal cells in the eye, J. Biomed. Opt. 22 (2017), 060504.
[105] H.G. Breunig, A. Uchugonova, A. Batista, K. König, Software-aided automatic laser optoporation and transfection of cells, Sci. Rep. 5 (2015) 1–11.
[106] H.G. Breunig, A. Uchugonova, A. Batista, K. König, High-throughput continuous flow femtosecond laser-assisted cell optoporation and transfection, Microsc. Res. Tech. 77 (2014) 974–979.
[107] A. Uchugonova, H.G. Breunig, A. Batista, K. König, Optical reprogramming of human cells in an ultrashort femtosecond laser microfluidic transfection platform, J. Biophotonics 9 (2016) 942–947.
[108] R. Xiong, et al., Laser-assisted photoporation: fundamentals, technological advances and applications. *Adv*, Phys. X (1) (2016) 596–620.
[109] Y. Xie, C. Zhao, An optothermally generated surface bubble and its applications, Nanoscale (2017), https://doi.org/10.1039/c7nr01360d.
[110] G.C. Messina, et al., Spatially, temporally, and quantitatively controlled delivery of broad range of molecules into selected cells through Plasmonic nanotubes, Adv. Mater. 27 (2015) 7145–7149.
[111] Q. Fan, W. Hu, A.T. Ohta, Efficient single-cell poration by microsecond laser pulses, Lab Chip 15 (2015) 581–588.
[112] S. Courvoisier, et al., Plasmonic Tipless pyramid arrays for cell Poration, Nano Lett. 15 (2015) 4461–4466.
[113] N. Saklayen, et al., Intracellular delivery using nanosecond-laser excitation of large-area Plasmonic substrates, ACS Nano 11 (2017) 3671–3680.
[114] Y.C. Wu, et al., Massively parallel delivery of large cargo into mammalian cells with light pulses, Nat. Methods 12 (2015) 439–444.

[115] T. Man, et al., Intracellular Photothermal delivery for suspension cells using sharp Nanoscale tips in microwells, ACS Nano 13 (2019) 10835–10844.
[116] Z. Lyu, et al., A universal platform for macromolecular Deliveryinto cells using gold nanoparticle layers via the Photoporation effect, Adv. Funct. Mater. (2016), https://doi.org/10.1002/adfm.201602036.
[117] E.Y. Lukianova-Hleb, D.S. Wagner, M.K. Brenner, D.O. Lapotko, Cell-specific transmembrane injection of molecular cargo with gold nanoparticle-generated transient plasmonic nanobubbles, Biomaterials 33 (2012) 5441–5450.
[118] R. Xiong, et al., Comparison of gold nanoparticle mediated photoporation: vapor nanobubbles outperform direct heating for delivering macromolecules in live cells, ACS Nano 8 (2014) 6288–6296.
[119] T. Pylaev, E. Vanzha, E. Avdeeva, B. Khlebtsov, Khlebtsov, N. a novel cell transfection platform based on laser optoporation mediated by au nanostar layers, J. Biophotonics 12 (2019) 1–12.
[120] L. Wang, et al., Using porous magnetic iron oxide nanomaterials as a facile photoporation nanoplatform for macromolecular delivery, J. Mater. Chem. B 6 (2018) 4427–4436.
[121] L. Raes, et al., Gold nanoparticle-mediated Photoporation enables delivery of macromolecules over a wide range of molecular weights in human CD4+ T cells, Crystals (2019), https://doi.org/10.3390/cryst9080411.
[122] L. Wayteck, R. Xiong, K. Braeckmans, S.C. De Smedt, K. Raemdonck, Comparing photoporation and nucleofection for delivery of small interfering RNA to cytotoxic T cells, J. Control. Release 267 (2017) 154–162.
[123] S. Kalies, et al., Characterization of the cellular response triggered by gold nanoparticle–mediated laser manipulation, J. Biomed. Opt. 20 (2015), 115005.
[124] G. Houthaeve, et al., Targeted perturbation of nuclear envelope integrity with vapor nanobubble-mediated photoporation, ACS Nano 12 (2018) 7791–7802.
[125] J. Wu, et al., Two-in-one platform for high-efficiency intracellular delivery and cell harvest: when a Photothermal agent meets a Thermoresponsive polymer, ACS Appl. Mater. Interfaces 11 (2019) 12357–12366.
[126] J. Wu, et al., Intracellular delivery platform for 'recalcitrant' cells: when polymeric carrier marries Photoporation, ACS Appl. Mater. Interfaces 9 (2017) 21593–21598.
[127] European Commission, Guidelines on good manufacturing practice specific to advanced therapy medicinal products. EudraLex the rules governing medicinal products in the European Union volume 4 good manufacturing, Practice 6 (2017).
[128] X. Du, et al., Advanced physical techniques for gene delivery based on membrane perforation, Drug Deliv. 25 (2018) 1516–1525.
[129] https://www.novartis.us/sites/www.novartis.us/files/kymriah.pdf, accessed the 05.11.2020.

Bioelectrochemistry 87 (2012) 236–243

Contents lists available at SciVerse ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

A brief overview of electroporation pulse strength–duration space: A region where additional intracellular effects are expected

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ARTICLE INFO

Article history:
Received 29 July 2011
Received in revised form 9 January 2012
Accepted 28 February 2012
Available online 14 March 2012

Keywords:
Electroporation
Cell system
Cell model
Intracellular electroporation
Apoptosis

ABSTRACT

Electroporation (EP) of outer cell membranes is widely used in research, biotechnology and medicine. Now intracellular effects by organelle EP are of growing interest, mainly due to nanosecond pulsed electric fields (nsPEF). For perspective, here we provide an approximate overview of EP pulse strength–duration space. This overview locates approximately some known effects and applications in strength–duration space, and includes a region where additional intracellular EP effects are expected. A feature of intracellular EP is direct, electrical redistribution of endogenous biochemicals among cellular compartments. For example, intracellular EP may initiate a multistep process for apoptosis. In this hypothesis, initial EP pulses release calcium from the endoplasmic reticulum, followed by calcium redistribution within the cytoplasm. With further EP pulses calcium penetrates mitochondrial membranes and causes changes that trigger release of cytochrome *c* and other death molecules. Apoptosis may therefore occur even in the presence of apoptotic inhibitors, using pulses that are smaller, but longer, than nsPEF.

1. Introduction

Electroporation is now widely accepted as a mechanistic hypothesis relevant to the response of cell membranes to large electric field pulses that rapidly increase the transmembrane voltage, $U_m(t)$, of cell membranes to a value where cell membrane conductance dramatically rises [1,2]. For essentially the same conditions this leads to molecular transport through cell membranes. In both cases, the changed behavior is often attributed to a burst of pore creation. Recently, attention has expanded to include EP of intracellular membranes, with the membranes of progressively smaller organelles experiencing EP as the external electric field magnitude is increased. Due to the large size of the the EP literature, we cite only a few papers that reflect existing effects and applications or new, future directions.

An approximate overview of electrical conditions (pulse durations and strengths) for cell and tissue EP-based responses is shown in Fig. 1. Here, "large pulse" indicates that supra-physiologic transmembrane voltages are created at some or all of the sites of a cell membrane. Although early work often focused on artificial planar bilayer membranes, the main motivation now is cell EP, both in vitro (research, biotechnology and industrial processing) and in vivo (clinical research and application). For a typical mammalian cell plasma membrane (PM) this means that U_m rises to at least 200 mV (and in most cases is significantly larger, often reaching 1 to 1.5 V), but the duration is also important (although sometimes invoked, there is no fixed "critical voltage", because pore creation involves a finite, not infinite, rate).

The pulse strength–duration map (Fig. 1) involves nine orders of magnitude in duration but only three orders of magnitude in strength. This is consistent with the response of cells depending strongly on strength, and relatively weakly on time. This in turn is consistent with the use of an absolute rate equation for pore creation in most electroporation theories employed in spatially distributed cell system models.

The rough guide of Fig. 1 emphasizes some conditions where EP occurs, sufficient to cause established EP effects or applications. Details of the effects and applications are relatively unimportant for this rough guide to electric field pulse strength–duration space. In Section **2** we thus provide only illustrative references and brief descriptions.

There are two rationales for considering strength–duration space. First, the two parameters, "strength" and "duration" of individual pulses are generally chosen by investigators in describing EP protocols. If more than one pulse is used, investigators state how many pulses are used, and report any change in pulse strength and duration during a multiple pulse protocol. Second, "strength" and "duration" are a traditional pair of electrical stimulus parameters. Even irregularly shaped pulses are generally referred to by investigators using the simple "strength" and "duration", with the implicit understanding that any detailed analysis should consider the actual pulse waveform.

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doi:10.1016/j.bioelechem.2012.02.007

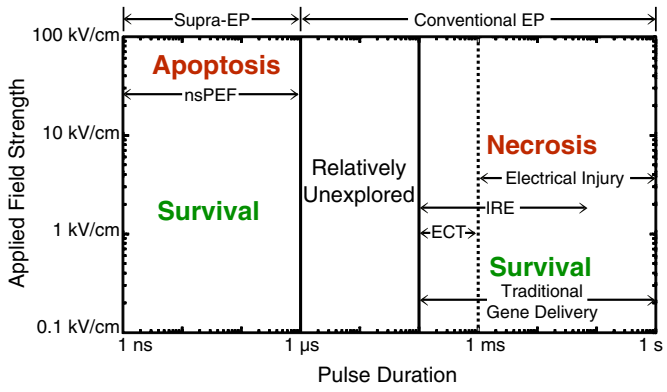

Fig. 1. Map of the approximate locations of effects and applications due to cell electroporation (EP) in pulse strength–duration space. The two-dimensional log–log display indicates approximate regions with well established effects and applications. This simple map is therefore a rough guide to much, but certainly not all, EP effects and applications. The vertical position of a label indicates an approximate, associated applied field strength ($|E_{app}|$, magnitude), and the horizontal position of a label indicates a characteristic range of pulse duration. Each phenomenon or application is described briefly in subsections below. The purpose of this figure is to direct attention to a "big picture" of EP, its effects, and applications. Particular attention is directed to the "Relatively Unexplored" region between 1 and 100 µs, where additional intracellular EP effects are expected.

However, such complications cannot be part of the simple, approximate map (Fig. 1). For similar reasons, in Section **2** we provide only brief illustrative descriptions and references.

1.1. Pulse duration

Pulse durations range from approximately 1 ns to almost 1 s (we consider individual pulse widths). Examples are given below in the brief descriptions of EP phenomena and applications.

1.2. Electric field strength

The strength or magnitude of applied electric field pulses range from approximately 100 kV/cm down to 0.1 kV/cm. Because tradition in the EP scientific and engineering literature favors units of V/cm we use these rather than SRI units (V/m). EP pulses of course generate Joule heating, but the pulse duration is small enough that, generally, the temperature rise can be regarded as negligible, at least in terms of causing the main response (membrane pore creation). A temperature rise of a few Kelvin (or °C) does not by itself create significant poration.

2. Effects and applications

2.1. Supra-EP

Exposure to very large fields with durations less than a cell's passive plasma membrane (PM) charging time result in much more numerous, but smaller-sized, pores than conventional electroporation [3] (see below). For the largest fields employed, pores are created in almost all regions of a cell's membranes, including those of small organelles such as mitochondria. One result is that the redistributed electric field mostly goes through cells (isolated or members of a tissue) [4–8]. This behavior is the opposite to that of conventional EP responses. There is now strong experimental [9–11] and theoretical [7] support for the interpretation that nsPEF leading to supra-EP involves ionic conduction currents through pores.

However, the distinction between supra- and conventional-EP is somewhat arbitrary: there is no sudden change in behavior as field strength is increased and pulse duration is shortened. Instead, a continuous transition between the two extremes is expected (see "Relatively Unexplored" region of Fig. 1).

2.2. Conventional electroporation

The overwhelming majority of EP investigations and applications involve conventional EP. These involve large pulses in the sense that the transmembrane voltage, U_m, rises to supra-physiologic values, typically at least 0.2 V, but more often near or greater than 1 V for some locations on a cell's PM. Conventional EP involves pulse durations longer than the passive PM charging time (often in the range 0.4 to 1 µs or more). The cell response involves a significant increase in PM conductance at some locations, but for most established conditions, the redistributed field largely passes around the cell [4–6,12–18].

2.3. nsPEF (nanosecond pulsed electric fields)

Since the publication of an attention-getting paper in 2001 that reported intracellular effects due to ultrashort pulses [19], the topic of cellular response to sub-microsecond pulses with mega-volt per meter magnitudes (i.e. 10 kV/cm) has included consideration of 'nanosecond' pulses, even as the pulse duration approaches 1000 ns (1 µs). A striking feature of cell killing by nsPEF is death by apoptosis [20,21]. This has motivated significant interest in nsPEF as a basis for treating cancer tumors without delivering drugs or genes [20,22–25].

2.4. Electrical injury

Although electrical injury often has a major thermal component that burns tissue, significant and insidious non-thermal damage can also occur. A generally accepted hypothesis is cell death by irreversible EP, leading to necrosis [26–31].

In the case of ELF (extremely low frequency; widely used 50–60 Hz is included) the larger cells (skeletal muscle, nerve) are the most vulnerable. The characteristic "pulse" associated with these periodic fields is taken here to be a half (~10 ms) or quarter cycle (~5 ms). An important insight is that within the human body, large cells (e.g. skeletal muscle, nerve cells) are particularly vulnerable to these relatively long-lasting strong fields, which are in the conventional EP category. The significant differential vulnerability arises from the disparity in cell size, due to membrane-based field amplification (voltage concentration) [32,33]. In the case of conventional EP, larger cells more readily electroporate than smaller cells for the same magnitude field. Thus, electrical shock injury can involve EP-mediated lysis with or without an elevated temperature from Joule heating, and appears to be consistent with necrotic cell death due to loss of PM integrity.

2.5. IRE (irreversible electroporation)

IRE is closely related to non-thermal electrical shock injury, but is a purposeful, controlled intervention aimed at tumor treatment [34–41]. These and other studies strongly suggest that IRE is likely to be a highly effective method for tumor ablation. Like all EP-based methods, it does not lose effectiveness at tumor sites near thermally significant blood vessels that provide cooling, and therefore can treat cancer cells that might otherwise survive thermal ablation methods. As in the case of electrical injury, necrotic cell death dominates. Aggressive pursuit of clinical applications is ongoing.

2.6. ECT (electrochemotherapy)

This well established EP-based approach to tumor treatment involves shorter, smaller pulses that typically cause reversible EP, with tumor treatment based on delivery of potent anticancer drugs (usually bleomycin) into cells [42–49]. Typically, eight square (trapezoidal) pulses of duration 100 µs at 1 s intervals are used. Without bleomycin present, cells survive, but effective tumor ablation occurs

if small (nM) extracellular concentrations of bleomycin are provided. ECT is becoming widely used [50], and continues to grow in clinical importance.

2.7. In vivo gene delivery

In vivo delivery of large DNA molecules into cells of viable tissue [51] involves reversible EP created by still smaller and longer pulses (Fig. 1). Often, modification of skeletal muscle or dendritic cells is sought, leading to significantly improved tumor treatment and, perhaps most importantly, enhanced vaccination overall [52–56]. Relatively small field pulses with longer durations combined with small, long electric field pulses (to electrophoretically mediate transport of highly charged and electrically mobile DNA) and other multiple pulse protocols are commonly employed. This medical and biotechnological application, which relies on cell survival rather than death, is receiving considerable attention and has the prospect of making a major impact.

The delivery of oligonucleotides by EP is also of growing interest [57,58]. In this case, the highly charged molecules may have a greater probability of being delivered through the PM, with less interception and binding to the membrane prior to stepwise entry into the cell [59].

2.8. Relatively unexplored region: site of new intracellular effects?

This region in Fig. 1 is somewhat arbitrarily defined by pulse durations from 1 to 100 μs (two of the nine orders of magnitude in pulse duration), but is meant to point to a potentially useful range of pulse strengths and duration yet to be fully investigated. It is located between the declared 1000 ns upper limit for nsPEF and the 100 μs lower boundary of most ECT and IRE pulse protocols. However, the designation "Relatively Unexplored" means only that relatively few publications involve these electric field pulses.

Pulses within this region may be well suited to manipulating cells by intracellular-EP, viz. by in situ electroporation of organelle membranes. A recent theory-based cell system modeling paper makes explicit estimates supporting this possibility [18]. Two mechanisms are suggested: (1) Use of sufficiently large (2 to 9 kV/cm exponentially-decaying pulses with 40 μs time constant to cause EP in progressively smaller organelles (endoplasmic reticulum membrane to nuclear envelope membranes to outer and inner mitochondrial membranes) as pulse strength is increased. (2) Use of a 2 ms trapezoidal pulse (outside the Relatively Unexplored region) that generates sufficient PM poration that intracellular fields raise transmembrane voltages high enough to gate organelle channels. Thus, some pulses within this region should cause intracellular effects without using nsPEF, suggesting that unusually large conventional EP pulses can be used instead.

2.9. Important prior examples in the "Unexplored Region"

A few examples are given below. In the future there will likely be more, based on more widely ranging research, including both experiments and theory-based cell system models.

Several early experiments (we cite two of eight [60,61]) with human erythrocytes used pulses with strength and duration that belong to the Relatively Unexplored region of Fig. 1. Since mature (terminally differentiated) human erythrocytes have no organelles [62], intracellular EP would not have been possible. Accordingly, while there were important insights into PM EP there was no hint of intracellular effects.

A first example using cells with organelles is the tremendously influential work that first demonstrated uptake and expression of DNA using mouse cells [63]. This early experiment used "exponential pulses" (exponentially decaying pulses) with time constant 5 μs, applying 3 pulses at 10 min intervals at 20 °C. DNA transfection was observed over the range 6 to 10 kV/cm, with maximum effect at 8 kV/cm. This pulse selection lies in the Relatively Unexplored region. Presently, longer (often millisecond) pulses with smaller strengths are predominantly used for DNA transfection protocols. One reason is that smaller pulses can be more readily generated with simpler pulse generators.

A second example is the use of exponential pulses with a time constant of 40 μs and strengths ranging from 4.5 to 8.1 kV/cm [64]. This study reports several indicators of cell death by apoptosis, yet the electrical conditions (duration and strength) are well outside the nsPEF region. The authors of this important study proposed an explanation based on salt composition and ionic strength of the extracellular medium. As suggested above, an alternative explanation is intracellular EP.

A third important example is "Nucleofection", which uses pulses to deliver DNA directly into the nucleus of eukaryotic cells. Information regarding pulse strength and duration is more readily found in the patent literature than in research publications [65–67], which typically cite program protocols for pulsing designed by the manufacturer, and are not described in detail. In one general application, pulses are applied in pairs, with an initial large and relatively short pulse (2–10 kV/cm, 10–200 μs) immediately followed by a smaller but longer pulse of 100 ms maximum duration [68]. Alternatively, a train of 1 to 10 pulses with rest intervals of at least 100 μs is applied, with each pulse having a duration of 10 μs to 5 ms and field strengths varying from 1 to 10 kV/cm [69]. Nucleofection is likely to involve essentially simultaneous EP of the PM and membranes of the nuclear envelope, and regions of the endoplasmic reticulum (ER) membrane are also likely to be electroporated, which could lead to calcium release into the cytoplasm.

2.10. Future examples

Additional EP phenomena and effects may await discovery. For the stronger pulses in this region, behavior similar to that of supra-EP should be expected. Specifically, more extensive EP is expected to occur, with increased membrane conductance at the very sites that have larger membrane resistance. This means that cell membrane barriers will tend to be greatly decreased, both for the outer (plasma) membrane and progressively for inner (organelle) membranes as pulse field strength is increased.

In view of recent and ongoing improvements in cell system modeling, we argue that a combined computational and experimental approach to research can be rewarding. In silico cell models can be used to explore pulse parameter space more rapidly than experiments alone. We note two examples. In one, an isolated cell model has demonstrated progressive EP of intracellular (organelle) membranes, with smaller and smaller organelles experiencing EP as pulse strength is increased for an exponential pulse with a 40 μs time constant [18]. In the second example, the same cell system model was used, but a digitized experimental nsPEF pulse waveform [24] was used; this digitized irregular waveform was also applied to an in vivo multicellular model [70].

3. Cell system models and intracellular effects

3.1. General features of cell models

Cell models translate mathematically described field pulses into electrical responses. Formally, these responses include spatially and temporally distributed transmembrane voltages, U_m, in addition to electrical currents within the aqueous electrolytes of a cell model's compartments. Pore creation, pore size evolution, and pore transport properties can also be included. These responses are governed by the time-dependent value of U_m at local membrane sites. However, the

dynamic pore populations are also the result of local history, as pores accumulate and eventually vanish differently at membrane sites with different electrical and poration histories.

Solutions to cell system models can therefore be complicated. At any time point, the response is the result of interactions between all of the local models within the cell system model. This means that membrane EP behavior can also be emergent and non-intuitive. The ultimate test, however, is whether the models generate responses that can be tested by comparison with experiments. And this is what is important here: what existing empirical evidence and future experimental results will determine whether intracellular effects occur in this new pulse strength–duration region of interest?

3.2. Models with several different organelles

Although there are a number of valuable cell system models that describe some aspects of cell EP quantitatively, only a few [4,6,7,18,70] contain several different organelles and the requisite single or double membranes with idealized or irregular geometry along with appropriate resting potential sources. These models were originally motivated by the challenge of understanding cell responses to both nsPEF and conventional EP pulses. However, these same models can be examined for their responses to other pulses (waveforms and strengths). This is a fundamental attribute of cell system models: a response to almost any pulse can be observed.

3.3. Model responses to nsPEF and conventional EP

A broad finding is that while conventional EP pulses almost exclusively alter the PM, the much stronger nsPEF pulses with submicrosecond durations create many small ion conducting pores early in the pulse [7]. This facilitates massive small ion transport through cell membranes. Once a burst of pore creation occurs, transmembrane currents shift from displacement currents to conduction currents. This breach of the PM means that large intracellular fields can be maintained for a long time. Thus, large fields with durations longer than 1 μs are candidates for causing intracellular effects by organelle EP.

Instead of moving small ions to charge a local membrane area, small ions now move through the membrane by entering and exiting the numerous small pores (conduction current). These large ionic conduction currents pervade the cell, creating large intracellular fields. In the case of nsPEF, as pulse magnitudes increase, progressively smaller organelles are electroporated, and the intracellular electric field becomes progressively more spatially uniform.

3.4. Model used to show intracellular EP beyond nsPEF

A cell system model based on a 2D Cartesian transport lattice has been recently used to quantitatively describe some responses to large conventional EP pulses (durations longer than one microsecond). Construction and solution of this model are described elsewhere [18].

This first analysis [18] used this cell system model with a 40 μs exponential pulse (1 μs rise time) for several different applied field strengths (e.g. 1, 2, 4 and 7 kV/cm). At an applied field strength of 1 kV/cm, only the polar regions of the PM are electroporated, with slight asymmetry (due mostly to the resting potential source, and partially to irregularly located organelles). At 2 kV/cm the electroporated polar region has expanded, and some of the ER is involved, but with different, unconnected local ER membrane areas now porated. At this point, neither the nuclear membranes nor the mitochondrial membranes have significant pores. By 4 kV/cm the electroporated polar region has expanded further: most of the ER and about half of the nuclear membranes is also electroporated. Finally, upon reaching 7 kV/cm EP has become widespread within the organelle membranes of the model, including the mitochondria. The motivation for considering this particular pulse is an experiment that reported apoptosis, but with an explanation based on the composition and concentration (ionic strength) of the media used [64]. We have instead proposed intracellular EP as an explanation.

A second analysis [70] used the same model with a relatively large IRE trapezoidal pulse (duration of 100 μs and 10 μs rise/fall times, and strength: $E_{pp} = 2.5$ kV/cm). The pulse reaches its maximum value in 1 μs, and after a total of 99 μs the field linearly decreases, reaching zero at 100 μs. In this case we examined the model's response at four different times.

At 10 μs the PM has electroporated near the poles, with slight asymmetry. Several extended sites within the ER have already been significantly electroporated, but the nuclear envelope and mitochondria have not. At 20 μs the number of local ER membrane sites with significant pores has increased only slightly, and the other organelles have not been electroporated. At 99 μs there is actually a small decrease in porated sites. This model behavior emerges because after an initial burst of pore creation early in the pulse, U_m falls from a peak value of ~1.2 V to about ~0.5 V, a value at which pore creation is relatively small. Simultaneously, however, pores are decaying with an assumed mean lifetime of 3 ms, and this accounts for a slight (about 3%) loss of pores at 99 μs. Overall, the model's response to this pulse includes some ER electroporation, potentially to a degree sufficient to release significant amounts of calcium from ER stores.

3.5. Inescapable Joule heating

Although EP itself is a non-thermal response, the application of electric fields unavoidably causes some dissipation (Joule heating). Generally, while it is recognized that this companion process is inescapable, a modest temperature rise alone causes insignificant poration. Nevertheless, very large and/or long pulses can create temperature rises which lead to non-specific cumulative thermal damage [71]. The simplest, most conservative estimate is the adiabatic approximation, which simply assumes that no heat transfer occurs on the time scale of interest.

It is reported that vascular block [72] or blood flow cessation [22,24] occurs for some electroporating pulses. If normal perfusion exists, then the Pennes bioheat equation provides quantitative guidance for the temperature rise. Often, thermal damage is estimated by computing an indicator quantity. This is basically an absolute rate estimate that is applied, subject to the condition that only temperature excursions above 42 °C are included. Below 42 °C, however, it is assumed that biological repair mechanisms are adequate. With these considerations in mind, we present estimates of the temperature achieved for simple trapezoidal pulses of 1, 3, 10, 30 and 100 μs duration and 1 μs rise/fall times (Tables 1 and 2; see Appendix A).

3.6. Models are hypotheses

An important simple fact should be kept in mind: Models are hypotheses [73]. These hypotheses may be relatively complicated, but they can be quantitatively tested. Nevertheless, the responses are those of a model, not a real cell. Their value lies in providing insights more readily, and more rapidly, than by use of experiments alone. The primary response of EP is basically biophysical, presently approximated as emerging solely in small lipid bilayer regions of a cell membrane, which with its many proteins is highly complex [74].

3.7. Non-thermal cell death by electric fields

Here we illustrate the potential significance of intracellular EP generally by considering the outstanding problem of understanding the mechanism(s) of cell death by electric fields. Here we focus on a hypothesis involving intracellular EP.

It has long been known that large electric field pulses used for EP can kill cells without heating being the primary cause [30,34,75,76]. In fact, EP is the basis of ongoing approaches to local cancer tumor treatment in which neither drugs nor genes are introduced. Chronologically, the first approach is based on nsPEF, which generally kills cells by apoptosis [20,24,77]. The second approach is based on IRE, which generally causes cell death by necrosis [34,37,78]. Strikingly, the largest strength fields apparently kill by apoptosis, while the smaller (but still large) fields kill by necrosis.

But how could bigger lethal fields allow cells to have more control over their own demise: apoptosis rather than necrosis? The illustrative hypothesis presented below features a multistep process using two sets of EP pulses. It builds on biochemical processes that regulate the mitochondrial apoptosis pathway (Fig. 2) [79,80]. What is new in the illustrative hypothesis is the replacement of some biochemical stimuli with membrane EP stimuli.

In this hypothesis, initial EP pulses release calcium from the endoplasmic reticulum, followed by a delayed calcium redistribution within the cytoplasm. Calcium can also enter from the extracellular space by PM EP. With further EP pulses calcium penetrates mitochondrial membranes and causes mitochondrial disruption that releases cytochrome *c* and other death molecules such as SMAC/Diablo, EndoG, and AIF. Apoptosis may therefore occur even in the presence of molecular inhibitors, due to nsPEF or pulses in

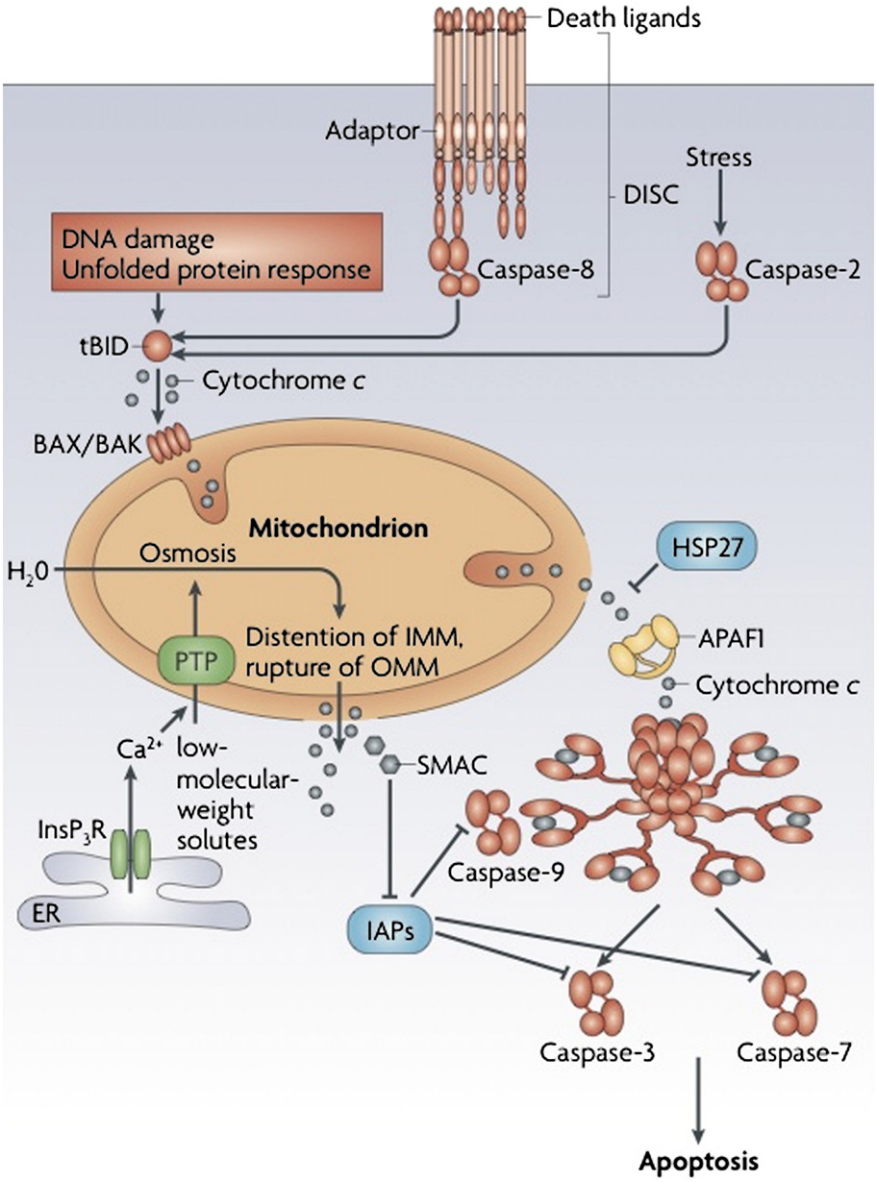

Fig. 2. Apoptosis biochemical processes and stimuli [80]. Under normal conditions, pro-apoptotic inner mitochondrial membrane proteins, such as cytochrome *c* (gray dots) and second mitochondria-derived activator of caspase (SMAC, also known as Diablo; grey hexagons) remain sequestered in the mitochondria. Following an apoptotic stimulus (such as DNA damage, unfolded protein response or stress), BH3 interacting-domain death agonist (BID) is cleaved to yield truncated (t)BID. tBID in turn activates Bcl-2 associated proteins BAX and BAK by inducing their oligomerization to form pores in the outer mitochondrial membrane (OMM). Cytochrome *c* exits the mitochondrion through these pores, the permeability transition pore (PTP) and/or other channels. Once in the cytosol, cytochrome *c* binds to apoptotic protease-activating factor-1 (APAF1), enabling its heptamerization and binding to procaspase-9. Activated caspase-9 then activates the executioner caspases-3 and -7. Feedback loops that might promote the release of cytochrome *c* also exist, such as the opening of the PTP by Ca^{2+} that is released from the endoplasmic reticulum (ER). Opening of the PTP promotes influx of low-molecular-weight solutes into the mitochondria. To counter the pro-apoptotic events, cytochrome *c* release can be inhibited by heat-shock protein-27 (HSP27), whereas the caspases can be inhibited by the inhibitors of apoptosis proteins (IAPs). IAPs are inhibited by SMAC. DISC, death-inducing signaling complex; $InsP_3$ R, inositol-1,4,5-triphosphate receptor. Reproduced with permission from Nature Rev. Mol. Cell. Biol. [80].

the Relatively Unexplored region that are smaller, but longer, than nsPEF.

Fig. 2 is reproduced from a 2008 review of biochemical and membrane events for cytochrome *c* release and apoptosis initiation [80]. Another, very comprehensive review of biochemically-induced apoptosis also emphasizes the important role of cell membranes, both the outer membrane (plasma membrane or PM) and inner, organelle membranes [79]. The illustrative intracellular EP hypothesis unavoidably involves the PM, but explicitly also involves EP of the endoplasmic reticulum (ER) membrane, and both the OMM and IMM (outer- and inner mitochondrial membranes). Below we briefly discuss the key EP features of the hypothesis.

3.8. Details of the hypothesis

A hypothetical protocol begins with a first set of one or more pulses causing EP in the ER membrane, and unavoidably also in the PM. Ca^{2+} then enters the cytoplasm from two sources, the stores within the ER and the extracellular medium. The newly arrived Ca^{2+} then redistributes within the cytoplasm. During a pulse Ca^{2+} redistribution occurs by electrodiffusion. Between pulses redistribution is by diffusion. With time, when the cytosolic Ca^{2+} concentration is sufficiently elevated, additional pulses capable of electroporating both the OMM and IMM (outer- and inner mitochondrial membranes) are applied. This second pulse set delivers Ca^{2+} into both the IMS (intermembrane space) and into the mitochondrial cristae.

The first set of pulses need only release Ca^{2+} from the ER (and in part Ca^{2+} from the extracellular space via the PM), so pulse strength need be only moderately large [18]. In this case, the second pulse set uses greater strength pulses to achieve EP in the OMM and IMM of the smaller mitochondria [18]. For very large pulses only one pulse set is needed, as pulses large enough to cause mitochondrial EP will also cause ER EP.

As shown in the lower left of Fig. 2 one key biochemical event is transfer of Ca^{2+} from the ER into mitochondria. Two pathways are possible: (I) through electropores in the OMM and IMM, and (II) through the PTP (permeability transition pore, a large protein-based structure). The PTP can gate into the open state if the IMM is depolarized about 100 s or longer [81], which may occur by IMM EP [82]. Low molecular weight (mass) solutes also enter the mitochondria interior.

In an additional role, delivered Ca^{2+} can also mobilize cytochrome *c* from its bound state to cardiolipin at the outer leaflet of the IMM. Remodeling of the matrix and cristae can also occur with small solute entry.

With time an osmotic influx of water swells and distends the IMM. Eventually, with enough time, the swelling leads to OMM rupture. At this time cytochrome *c*, and other death molecules are released into the cytoplasm; see Fig. 2 lower central region. For many cells this triggers irreversible initiation of apoptosis.

The net result is a two step process: (1) Ca^{2+} delivery from both the ER and extracellular space to, and into, mitochondria, and (2) IMM distension leading to OMM rupture and release of death molecules (cytochrome *c*, SMAC, others). Thus, the illustrative hypothesis suggests how intracellular EP might lead to additional effects and applications.

3.9. Intracellular EP of organelles smaller than mitochondria

For both nsPEF [3] and smaller, longer pulses [18], relatively large pulses create such a large PM conductance that the intracellular field approaches the external field. This means that experiments on isolated organelles provide some guidance for intracellular EP conditions.

Very early studies of isolated chromaffin granules (from bovine medullary cells; radii $\approx$ 120 nm, much smaller than lysosomes) indicated that they could be electroporated at a field strength of 20 kV/cm and duration 150 μs (exponential pulse decay time constant) [83].

Table 1
In vitro temperature reached after pulse: Initial temperature is assumed to be 23 °C; electrical conductivity is 1.2 S/m.

Pulse duration (μs)	Field strength (kV/cm)				
	1.0	2.0	5.0	10	30
1.0	23	23	23	23	26 ± 2 °C
3.0	23	23	23	24	31
10	23	23	24	26	>42
30	23	23	25	32	>42
100	23	24	31	>42	>42

These granules are significantly smaller than mitochondria. However, the temperature rise must be considered (about 6 °C for the granules). Organelles somewhat larger than these granules should experience EP at smaller intracellular fields, and are therefore candidates.

Accordingly, we should also consider organelles smaller than mitochondria, such as lysosomes [84]. Lysosomes are known to play a role in cell death through lysosomal-membrane permeabilization [85]. Lysosome size range is broad [84], with radii of 500 nm to 0.6 μm. This suggests that lysosomes are candidates for intracellular EP while avoiding excessive temperatures. As suggested by this explicit, illustrative qualitative hypothesis, intracellular EP is worthy of further exploration.

4. Summary

Following the important 2001 paper [19] reporting experimental results with intracellular effects by megavolt per meter, submicrosecond pulses (now often simply "nsPEF"), there has been great interest in the nature of these effects. At the same time, there is corresponding interest in quantitative, mechanistic understanding of how these, and similar, effects are caused.

Here we provide an approximate strength–duration map for several well established EP effects and applications. We then discuss the results of two recent models that explicitly treat intracellular effects associated with single and double-membrane organelles, which are purposefully located at irregular locations within a cell and are characterized by membranes of both idealized (traditional) and irregular (more realistic) geometry. We conclude with presentation of an illustrative mechanistic hypothesis for non-thermal cell death by apoptosis that is based on intracellular EP.

Acknowledgments

Supported by NIH grant GM063857 to JCW, and Fellowships to KCS from the NSF and Harvard-MIT Division of Health Sciences and Technology. We thank P. T. Vernier, A. G. Pakhomov, R. Nuccitelli, E. Neumann, D. Miklavic, R. C. Lee and S. J. Beebe for many valuable discussions, and K. G. Weaver for continual computer support. We apologize to our many colleagues whose papers we did not cite in this brief overview.

Table 2
In vitro temperature reached after pulse: Initial temperature is assumed to be 37 °C; electrical conductivity is 0.2 S/m (six times smaller than the in vitro case; see caveat below).

Pulse duration (μs)	Field strength (kV/cm)				
	1.0	2.0	5.0	10	30
1.0	37	37	37	37	37 °C
3.0	37	37	37	37	38
10	37	37	37	38	42
30	37	37	37	39	>42
100	37	37	38	>42	>42

Appendix A

The application of electrical pulses leads to thermal dissipation by Joule heating. Multiple pulse protocols with very large or very long pulses may cause significant cumulative thermal damage. Here we estimate the adiabatic temperature increase from single electrical pulses of different field strength and duration.

$$T_f = T_i + \frac{\sigma}{c\rho}\left|E\right|^2 \Delta t \quad (1)$$

Here T_i and T_f are the initial and final temperatures in °C, respectively, σ is the electrical conductivity (1.2 S/m for saline; 0.2 S/m for tissue), c is the specific heat of saline (4 kJ/kg K), ρ is the density of saline (1000 kg/m^3), $|E|$ is the magnitude of the peak field strength in V/m, and Δt is pulse duration in s. The thermal properties are obtained from [86].

Estimated temperature achieved in vitro

Typical in vitro laboratory experiments with isolated cells are carried out at room temperature (not well defined; about 23 ± 2 °C). If the simple view of damage onset only at or above 42 °C is presumed, the maximum temperature rise allowable is about 19 °C. This example assumes that a thermostated exposure/pulsing system set to a higher temperature (e.g. 37 °C) is not employed. Based on the adiabatic approximation, a single pulse of different pulse durations causes the in vitro temperature to exceed 42 °C at the following field strengths: 95 kV/cm (1 μs), 48 kV/cm (3 μs), 25 kV/cm (10 μs), 14 kV/cm (30 μs), and 8 kV/cm (100 μs).

Estimated temperature achieved in vivo

Following the above in vitro example, for a temperature rise estimate under the in vivo condition we must reduce the allowable temperature achieved to 42 °C (assumes no febrile subjects), which means that a rise of only 5 °C triggers consideration of accumulated thermal damage (which in turn involves the time to cool to a lower temperature). A bigger rise is possible if the treated site is peripheral, e.g. near the surface of a human arm (~27 °C). Again based on the adiabatic approximation, a single pulse of different pulse durations causes the in vivo temperature to exceed 42 °C at the following field strengths: 50 kV/cm (1 μs), 25 kV/cm (3 μs), 13 kV/cm (10 μs), 8 kV/cm (30 μs), and 4 kV/cm (100 μs).

An important caveat is that the cells of an electroporated tissue will experience intracellular fields and ionic currents. For small fields and low frequencies the tissue conductivity is due to ionic currents that flow around cells, within the interstitial space. However, if PM EP occurs then ionic currents also flow through cells of a tissue. This expectation is supported by both experiment [87,88] and theory [8,16]. Based on what is known about isolated cell EP, an electroporated tissue's effective electrical conductivity is expected to increase non-linearly with field strength, to depend on pulse duration, and to exhibit memory (hysteresis). For conditions leading to intracellular EP a larger temperature rise is expected, and is likely to be significant.

The approximate threshold for accumulating thermal damage by membranes and tissue at supraphysiologic temperatures is generally accepted to be be 42 °C. Below 42 °C, biological repair mechanisms are assumed to prevent tissue injury [89].

References

[1] E. Neumann, A. Sowers, C. Jordan (Eds.), Electroporation and Electrofusion in Cell Biology, Plenum Press, New York, 1989.
[2] A. Pakhomov, D. Miklavcic, M. Markov (Eds.), Advanced electroporation techniques in biology and medicine, CRC Press, Boca Raton, 2010.
[3] D.A. Stewart, T.R. Gowrishankar, J.C. Weaver, Transport lattice approach to describing cell electroporation: use of a local asymptotic model, IEEE Trans. Plasma Sci. 32 (2004) 1696–1708.
[4] T.R. Gowrishankar, A.T. Esser, Z. Vasilkoski, K.C. Smith, J.C. Weaver, Microdosimetry for conventional and supra-electroporation in cells with organelles, Biochem. Biophys. Res. Commun. 341 (2006) 1266–1276.
[5] T.R. Gowrishankar, J.C. Weaver, Electrical behavior and pore accumulation in a multicellular model for conventional and supra-electroporation, Biochem. Biophys. Res. Commun. 349 (2006) 643–653.
[6] K.C. Smith, T.R. Gowrishankar, A.T. Esser, D.A. Stewart, J.C. Weaver, Spatially distributed, dynamic transmembrane voltages of organelle and cell membranes due to 10 ns pulses: predictions of meshed and unmeshed transport network models, IEEE Trans. Plasma Sci. 34 (2006) 1394–1404.
[7] K.C. Smith, J.C. Weaver, Active mechanisms are needed to describe cell responses to submicrosecond, megavolt-per-meter pulses: cell models for ultrashort pulses, Biophys. J. 95 (2008) 1547–1563.
[8] A.T. Esser, K.C. Smith, T.R. Gowrishankar, J.C. Weaver, Towards solid tumor treatment by nanosecond pulsed electric fields, Technol. Cancer Res. Treat. 8 (2009) 289–306.
[9] P.T. Vernier, Y. Sun, M.A. Gundersen, Nanoelectropulse-driven membrane perturbation and small molecule permeabilization, BMC Cell Biol. 7 (2006) 37-1–37-16.
[10] A.G. Pakhomov, R. Shevin, J.A. White, J.F. Kolb, O.N. Pakhomova, R.P. Joshi, K.H. Schoenbach, Membrane permeabilization and cell damage by ultrashort electric field shocks, Arch. Biochem. Biophys. 465 (2007) 109–118.
[11] A.G. Pakhomov, J.F. Kolb, J.A. White, R.P. Joshi, S. Ziao, K.H. Schoenbach, Long-lasting membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF), Bioelectromagnetics 28 (2007) 655–663.
[12] K.A. DeBruin, W. Krassowska, Modeling electroporation in a single cell: I. Effects of field strength and rest potential, Biophys. J. 77 (1999) 1213–1224.
[13] K.A. DeBruin, W. Krassowska, Modeling electroporation in a single cell: II. Effects of ionic concentration, Biophys. J. 77 (1999) 1225–1233.
[14] W. Krassowska, P.D. Filev, Modeling electroporation in a single cell, Biophys. J. 92 (2007) 404–417.
[15] S. Talele, P. Gaynor, Non-linear time domain model of electropermeabilization: response of a single cell to an arbitrary applied electric field, J. Electrostat. 65 (2007) 775–784.
[16] A.T. Esser, K.C. Smith, T.R. Gowrishankar, J.C. Weaver, Towards solid tumor treatment by irreversible electroporation: Intrinsic redistribution of fields and currents in tissue, Technol. Cancer Res. Treat. 6 (2007) 261–273.
[17] S. Talele, P. Gaynor, M.J. Cree, J. van Ekeran, Modelling single cell electroporation with bipolar pulse parameters and dynamic pore radii, J. Electrostat. 68 (2010) 261–274.
[18] A.T. Esser, K.C. Smith, T.R. Gowrishankar, Z. Vasilkoski, J.C. Weaver, Mechanisms for the intracellular manipulation of organelles by conventional electroporation, Biophys. J. 98 (2010) 2506–2514.
[19] K.H. Schoenbach, S.J. Beebe, E.S. Buescher, Intracellular effect of ultrashort pulses, Bioelectromagnetics 22 (2001) 440–448.
[20] S.J. Beebe, P.M. Fox, L.J. Rec, K. Somers, R.H. Stark, K.H. Schoenbach, Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: apoptosis induction and tumor growth inhibition, IEEE Trans. Plasma Sci. 30 (2002) 286–292.
[21] S.J. Beebe, P.M. Fox, L.J. Rec, L.K. Willis, K.H. Schoenbach, Nanosecond, high intensity pulsed electric fields induce apoptosis in human cells, FASEB J. 17 (2003) 1493–1495.
[22] R. Nuccitelli, U. Pliquett, X. Chen, W. Ford, R.J. Swanson, S.J. Beebe, J.F. Kolb, K.H. Schoenbach, Nanosecond pulsed electric fields cause melanomas to self-destruct, Biochem. Biophys. Res. Commun. 343 (2006) 351–360.
[23] E.B. Garon, D. Sawcer, P.T. Vernier, T. Tang, Y. Sun, L. Marcu, M.A. Gundersen, H.P. Koeffler, In vitro and in vivo and a case report of intense nanosecond pulsed electric fields as a local therapy for human malignancies, Int. J. Cancer 121 (2007) 675–682.
[24] R. Nuccitelli, K. Tran, S. Sheikh, B. Athos, M. Kreis, P. Nuccitelli, Optimized nanosecond pulsed electric field therapy can cause murine malignant melanomas to self-destruct with a single treatment, Int. J. Cancer 127 (2010) 1727–1736.
[25] W. Ren, S.J. Beebe, An apoptosis targeted stimulus with nanosecond pulsed electric fields (nsPEFs) in E4 squamous cell carcinoma, Apoptosis 16 (2011) 382–393.
[26] R.C. Lee, M.S. Kolodney, Electrical injury mechanisms: electrical breakdown of cell membranes, Plast. Reconstr. Surg. 80 (1987) 672–679.
[27] R.C. Lee, D.C. Gaylor, K. Prakah-Assante, D. Bhatt, D.A. Israel, Role of cell membrane rupture in the pathogenesis of electrical trauma, J. Surg. Res. 44 (1988) 709–719.
[28] W. Chen, R.C. Lee, Electromediated permeabilization of frog skeletal muscle cell membrane: effect of voltage-gated ion channels, Bioelectrochem. Bioenerg. 34 (1994) 157–167.
[29] G.S. Abramov, M. Bier, M. Capelli-Schellpfeffer, R.C. Lee, Alteration in sensory nerve function following electrical shock, Burns 22 (1996) 602–606.
[30] R.C. Lee, D. Zhang, J. Hannig, Biophysical injury mechanisms in electrical shock trauma, Annu. Rev. Biomedical. Eng. 2 (2000) 477–509.
[31] C.J. Cela, R.C. Lee, G. Lazzi, Modeling cellular lysis in skeletal muscle due to electric shock, IEEE Trans. Biomed. Eng. 58 (2011) 1286–1293.
[32] T. Kotnik, D. Miklavčič, Second–order model of membrane electric field induced by alternating external electric fields, IEEE Trans. Biomed. Eng. 47 (2000) 1074–1081.
[33] T.R. Gowrishankar, J.C. Weaver, An approach to electrical modeling of single and multiple cells, Proc. Natl. Acad. Sci. 100 (2003) 3203–3208.
[34] L. Miller, J. Leor, B. Rubinsky, Cancer cells ablation with irreversible electroporation, Technol. Cancer Res. Treat. 4 (2005) 699–705.

[35] R.V. Davalos, L.M. Mir, B. Rubinsky, Tissue ablation and irreversible electroporation, Ann. Biomed. Eng. 33 (2005) 223–231.
[36] J.F. Edd, L. Horowitz, R.V. Davalos, L.M. Mir, B. Rubinsky, In vivo results of a new focal tissue ablation technique: irreversible electroporation, IEEE Trans. Biomed. Eng. 53 (2006) 1409–1415.
[37] B. Al-Sakere, F. André, C. Bernat, E. Connault, P. Opolon, R.V. Dvalos, B. Rubinsky, L.M. Mir, Tumor ablation with irreversible electroporation, PLoS One (November 2007) e1135.
[38] R.E. Neal II, R.V. Davalos, The feasibility of irreversible electroporation for the treatment of breast cancer and other heterogeneous systems, Ann. Biomedical. Eng. 37 (2009) 2615–2625.
[39] E. Maor, A. Ivorra, B. Rubinsky, Non thermal irreversible electroporation: novel technology for vascular smooth muscle ablation, PLoS One 4 (2009) e4757.
[40] Y. Granot, A. Ivorra, E. Maor, B. Rubinsky, In vivo imaging of irreversible electroporation by means of electrical impedance tomography, Phys. Med. Biol. 54 (2009) 4927–4943.
[41] R.E. Neal II, J.H. Rossmeisl Jr., P.A. Garcia, O.I. Lanz, N. Henao-Guerrero, R.V. Davalos, Successful treatment of a large soft tissue sarcoma with irreversible electroporation (epub ahead of print), J. Clin. Oncol. 29 (2011) e372–e377.
[42] M. Okino, H. Mohri, Effects of a high-voltage electrical impulse and an anticancer drug on *in vivo* growing tumors, Jpn. J. Cancer Res. 78 (1987) 1319–1321.
[43] S. Orlowski, J. B. Jr., C. Paoletti, L.M. Mir, Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs, Biochem. Pharmacol. 37 (1988) 4727–4733.
[44] L.M. Mir, S. Orlowski, J. Belehradek, J. Teissie, M.P. Rols, G. Sersa, D. Miklavčič, R. Gilbert, R. Heller, Biomedical applications of electric pulses with special emphasis on antitumor electrochemotherapy, Bioelectrochem. Bioenerg. 38 (1995) 203–207.
[45] R. Heller, M. Jaroszeski, L.F. Glass, J.L. Messina, D.P. Rapport, R.C. DeConti, N.A. Fenske, R.A. Gilbert, L.M. Mir, D.S. Reintgen, Phase I/II trial for the treatment of cutaneous and subcutaneous tumors using electrochemotherapy, Cancer 77 (1996) 964–971.
[46] J. Gehl, T. Skovsgaard, L.M. Mir, Enhancement of cytotoxicity by electropermeabilization: an improved method for screening drugs, Anticancer Drugs 9 (1998) 319–325.
[47] R. Heller, D. Coppola, C. Pottinger, R. Gilbert, M.J. Jaroszeski, Effect of electrochemotherapy on muscle and skin, Technol. Cancer Res. Treat. 1 (2002) 385–392.
[48] A. Gothelf, L.M. Mir, J. Gehl, Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation, Cancer Treat. Rev. 29 (2003) 371–387.
[49] J. Gehl, P.F. Geertsen, Palliation of haemorrhaging and ulcerated cutaneous tumors using electrochemotherapy, Eur. J. Cancer Suppl. 4 (2006) 35–37.
[50] L.M. Mir, J. Gehl, G. Sersa, C.G. Collins, J.R. Garbay, V. B., et al., Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator™ by means of invasive or non-invasive electrodes, Eur. J. Cancer Suppl. 4 (2006) 14–25.
[51] R. Heller, M. Jaroszeski, A. Atkin, D. Moradpour, R. Gilbert, J. Wands, C. Nicolau, In vivo gene electroinjection and expression in rat liver, FEBS Lett. 389 (1996) 225–228.
[52] L. Heller, M.J. Jaroszeski, D. Coppola, C. Pottinger, R. Gilbert, R. Heller, Electrically mediated plasmid DNA delivery to hepatocellular carcinomas in vivo, Gene Ther. 7 (2000) 826–829.
[53] B. Ferraro, Y.L. Cruz, D. Coppola, R. Heller, Intradermal delivery of plasmid $VEGF_{165}$ by electroporation promotes wound healing, Mol. Ther. 17 (2009) 651–657.
[54] A.M. B-Brakhop, R. Heller, R. Draghia Akli, Electroporation for the delivery of DNA-based vaccines and immunotherapeutics: current clinical developments, Mol. Ther. 17 (2009) 585–592.
[55] B.D. Livingston, S.F. Little, A. Luxembourgh, B. Ellefsen, D. Hannaman, Comparative performance of a licensed anthrax vaccine versus electroporation based on delivery of a PA endoding DNA vaccine in rhesus macaques, Vaccine 28 (2010) 1056–1061.
[56] A. Donate, D. Coppola, Y. Cruz, R. Heller, Evaluation of a novel non-penetrating electrode for use in DNA vaccination, PLoS One 6 (2011) e19181.
[57] M. Joergensen, B. Agerholm-Laarsen, P.E. Nielsen, J. Gehl, Efficiency of cellular delivery of antisense peptide nucleic acid by electroporation depends on charge and electroporation geometry, Oligonucleotides 21 (2011) 29–37.
[58] A. Paganin-Gioannia, E.B. Bellard, J.M. Escoffrea, M.P. Rols, J. Teissi, M. Golzio, Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells, Proc. Natl. Acad. Sci. 108 (2011) 10443–10447.
[59] E. Neumann, S. Kakorin, I. Tsoneva, B. Nikolova, T. Tomov, Calcium-medicated DNA adsorption to yeast cells and kinetics of cell transformation by electroporation, Biophys. J. 71 (1996) 868–877.
[60] K. Kinosita Jr., T.Y. Tsong, Formation and resealing of pores of controlled sizes in human erythrocyte membrane, Nature 268 (1977) 438–441.
[61] K. Kinosita Jr., T.Y. Tsong, Voltage-induced conductance in human erythrocyte membranes, Biochim. Biophys. Acta 554 (1979) 479–497.
[62] B.U. Samuel, N. Mohandas, T. Harrison, H. McManus, W. Rosse, M. Reid, K. Haldari, The role of cholesterol and glycosylphosphatidylinositol-anchored proteins of erythrocyte rafts in regulating raft protein content and malarial infection, J. Biol. Chem. 276 (2001) 29319–29329.
[63] E. Neumann, M. Schaefer-Ridder, Y. Wang, P.H. Hofschneider, Gene transfer into mouse lyoma cells by electroporation in high electric fields, EMBO J. 1 (1982) 841–845.
[64] F. Hofmann, L.H. Scheller, W. Strupp, U. Zimmermann, C. Jassoy, Electric field pulses can induce apoptosis, J. Membr. Biol. 169 (1999) 103–109.
[65] M. Zeitelhofer, J.P. Vessey, Y. Xie, F. Tübing, S. Thomas, M. Kiebler, R. Dahm, High-efficiency transfection of mammalian neurons via nucleofection, Nat. Protoc. 2 (7) (2007) 1692–1704.
[66] S.Y. Han, W. Gai, M. Yancovitz, I. Osman, C.J.D. Como, D. Polsky, Nucleofection is a highly effective gene transfer technique for human melanoma cell lines, Exp. Dermatol. 17 (2008) 405–411.
[67] T. Stroh, U. Erben, A.A. Kühl, M. Zeitz, B. Siegmund, Combined pulse electroporation – a novel strategy for highly efficient transfection of human and mouse cells, PloS One 5 (2010) e9488.
[68] H. Mueller-Hartmann, G. Riemen, K. Rothmann-Cosic, C. Thiel, L. Altrogge, M. Weigel, R. Christine, E. Lorbach, J. Helfrich, H. Wessendorf, G. Siebenkotten, Circuit arrangement for injecting nucleic acids and other biologically active molecules into the nucleus of higher eucaryotic cells using electrical current, U.S. Pub No.US 2009/0023131 A1, January 22, 2009.
[69] H. Mueller-Hartmann, M. Habig, Method for treating small volumes with electrical current, U.S. Patent No.7,700,357 B2, April 20, 2010.
[70] T.R. Gowrishankar, A.T. Esser, K.C. Smith, R.S. Son, J.C. Weaver, Intracellular electroporation site distributions: Modeling examples for nsPEF and IRE pulse waveforms, Proc. 33rd Ann. Int. Conf. IEEE EMBS Boston, MA, Aug 30–SEP 3, 2011, 2011, pp. 732–735.
[71] F.C. Henriques, Studies of thermal injury. V. the predictability and the significance of thermally induced rate processes leading to irreversible epidermal injury, Arch. Pathol. 43 (1947) 489–502.
[72] J. Gehl, T. Skovsgaard, L.M. Mir, Vascular reactions to in vivo electroporation: characterization and consequences for drug and gene delivery, Biochim. Biophys. Acta 1428 (1999) 233–240.
[73] B.M. Slepchenko, J.C. Schaff, I. Macara, L.M. Loew, Quantitative cell biology with the virtual cell, Trends Cell Biol. 13 (2003) 570–576.
[74] D.M. Engleman, Membranes are more mosaic than fluid, Nature 438 (2005) 578–580.
[75] D.C. Gaylor, K. Prakah-Asante, R.C. Lee, Significance of cell size and tissue structure in electrical trauma, J. Theor. Biol. 133 (1988) 255–259.
[76] B. Rubinsky, Irreversible electroporation in medicine, Technol. Cancer Res. Treat. 6 (2007) 255-159.
[77] P.T. Vernier, Y. Sun, L. Marcu, S. Salemi, C.M. Craft, M.A. Gundersen, Calcium bursts induced by nanosecond electric pulses, Biochem. Biophys. Res. Commun. 310 (2003) 286–295.
[78] P.A. Garcia, J.H. Rossmeis Jr., R.E. Neal II, T.L. Ellis, R.V. Davalos, A parametric study delineating irreversible electroporation from thermal damage based on a minimally invasive intracranial procedure, Biomed. Eng. Online 10 (2011) 34.
[79] G. Kroemer, L. Galluzzi, C. Brenner, Mitochondrial membrane permeabilization in cell death, Physiol. Rev. 87 (2007) 99–163.
[80] Y.-L.P. Ow, D.R. Green, Z. Hao, T.W. Mak, Cytochrome c: functions beyond respiration, Nat. Rev. Mol. Cell Biol. 9 (2008) 532–542.
[81] C. Loupatatzis, G. Seitz, P. Schonfeld, F. Lang, D. Siemen, Single-channel currents of the permeability transition pore from the inner mitochondrial membrane of rat liver and of a human hepatoma cell line, Cell. Physiol. Biochem. 12 (2002) 269–278.
[82] J.C. Weaver, Electroporation of biological membranes from multicellular to nano scales, IEEE Trans. Dielect. Electr. Insul. 10 (2003) 754–768.
[83] E. Neumann, K. Rosenheck, Permeability changes induced by electric impulses in vesicular membranes, J. Membr. Biol. 10 (1972) 279–290.
[84] E. Holtzmann, Lysosomes, Plenum Press, New York, 1989.
[85] G. Kroemer, M. Jäättelä, Lysosomes and autophagy in cell death control, Nat. Rev. Cancer 5 (2005) 866–897.
[86] R.V. Davalos, B. Rubinsky, Temperature considerations during irreversible electroporation, Int. J. Heat Mass Transfer 51 (2008) 5617–5622.
[87] R.V. Davalos, B. Rubinsky, L.M. Mir, D.M. Otten, Electrical impedance tomography for imaging tissue electroporation, IEEE Trans. Biomed. Eng. 51 (2004) 761–767.
[88] A. Ivorra, B. Al-Sakere, B. Rubinsky, L.M. Mir, In vivo electrical conductivity measurements during and after tumor electroporation: conductivity changes reflect the treatment outcome, Phys. Med. Biol. 54 (2009) 5949–5963.
[89] R.C. Lee, G. Russo, G. Kicska, Kinetics of heating in electrical shock, in: R.C. Lee, M. Capelli-Schellpfeffer, K.M. Kelly (Eds.), Electrical Injury: A Multidisciplinary Approach to Therapy, Prevention, Vol. 720, N.Y. Acad. Sci, New York, 1994, pp. 56–64.