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ARTICLE *in* NEW BIOTECHNOLOGY · JANUARY 2014

Impact Factor: 2.9 · DOI: 10.1016/j.nbt.2013.07.001 · Source: PubMed

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New Biotechnology • Volume 31, Number 1 • January 2014

Review

Engineering nucleases for gene targeting: safety and regulatory considerations

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Nuclease-based gene targeting (NBGT) represents a significant breakthrough in targeted genome editing since it is applicable from single-celled protozoa to human, including several species of economic importance. Along with the fast progress in NBGT and the increasing availability of customized nucleases, more data are available about off-target effects associated with the use of this approach. We discuss how NBGT may offer a new perspective for genetic modification, we address some aspects crucial for a safety improvement of the corresponding techniques and we also briefly relate the use of NBGT applications and products to the regulatory oversight.

Contents

Introduction

The last two decades, the improvement of organisms to meet agricultural, medical, industrial or environmental needs has been at least partly driven by the development of methods of stable

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1871-6784/$ - see front matter http://dx.doi.org/10.1016/j.nbt.2013.07.001

genetic modification. Mostly focused on the capacity and efficacy to introduce foreign genetic material (nucleic acid material from a non crossable or sexually incompatible organism), these methods often bear the burden of lack of control as regards the site of modification. The random introduction of a genetic modification could lead to unintended effects (alterations in the organisms' genome which go beyond the intended effect(s) of the genetic modification) due to insertional mutagenesis or position-effects. Hence modification of the genome in a targeted way offers interesting opportunities in terms of specificity and predictability.

The first methods for homologous gene targeting were described in yeast [1–5]. The inherently low efficiency in higher eukaryotes has prevented it from being used as a routine protocol, although very effective methods have been developed for mouse embryonic stem (ES) cells [6]. Targeted gene modification, including mutagenesis, has also been achieved by the use of oligonucleotides [7], small DNA fragments [8], pseudo-complementary peptide nucleic acids [9] or adeno-associated virus vectors [10]. A literature review of the last 5 years shows that the development of nuclease-based gene targeting (NBGT) has made remarkable progress, enabling modification (point mutation, deletion or insertion) at a genomic site of choice in various cell types and organisms (Fig. 1) [11–59]. Engineered and targetable nucleases are amongst others explored as tools for genetic modification of economically important crops [60–62] and animals [13], as alternative therapeutic strategies for infectious diseases [63–65] and as a method for human gene therapy as exemplified by three phase I clinical trials aiming at treating HIV/AIDS patients [66–68]. Three classes of nucleases are currently most used: Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and meganucleases (also called homing endonucleases). Moreover, a novel system, called CRISPR (for 'Clustered Regularly Interspersed Short Palindromic Repeats')/Cas has been developed recently as an additional approach for NBGT. These engineered nucleases create a double-strand break (DSB) which is either repaired inaccurately by non-homologous end joining (NHEJ), an error prone process often creating insertions or deletions (indels) or, when a donor DNA is provided, can be repaired by homologous recombination (HR), thereby mediating the exchange of the target with the donor sequence (Fig. 2).

Engineering nucleases

Zinc-finger nucleases

ZFNs are hybrid proteins with a DNA-recognition domain made up of zinc fingers (ZFs) and a DNA-cleavage domain coming from the *Fok*I restriction endonuclease (Fig. 3) [69]. Each ZF interacts mainly with 3 base pairs (bp) of DNA and engineered fingers have been combined to recognize 9-, 12-, 15- or 18-bp DNA sequences. Because the *Fok*I domain must dimerize to be active, a pair of ZFN monomers is designed for sequences separated by a 5- or 6-bp spacer. When both members of the pair bind, the cleavage domains dimerize and create a DSB within the spacer.

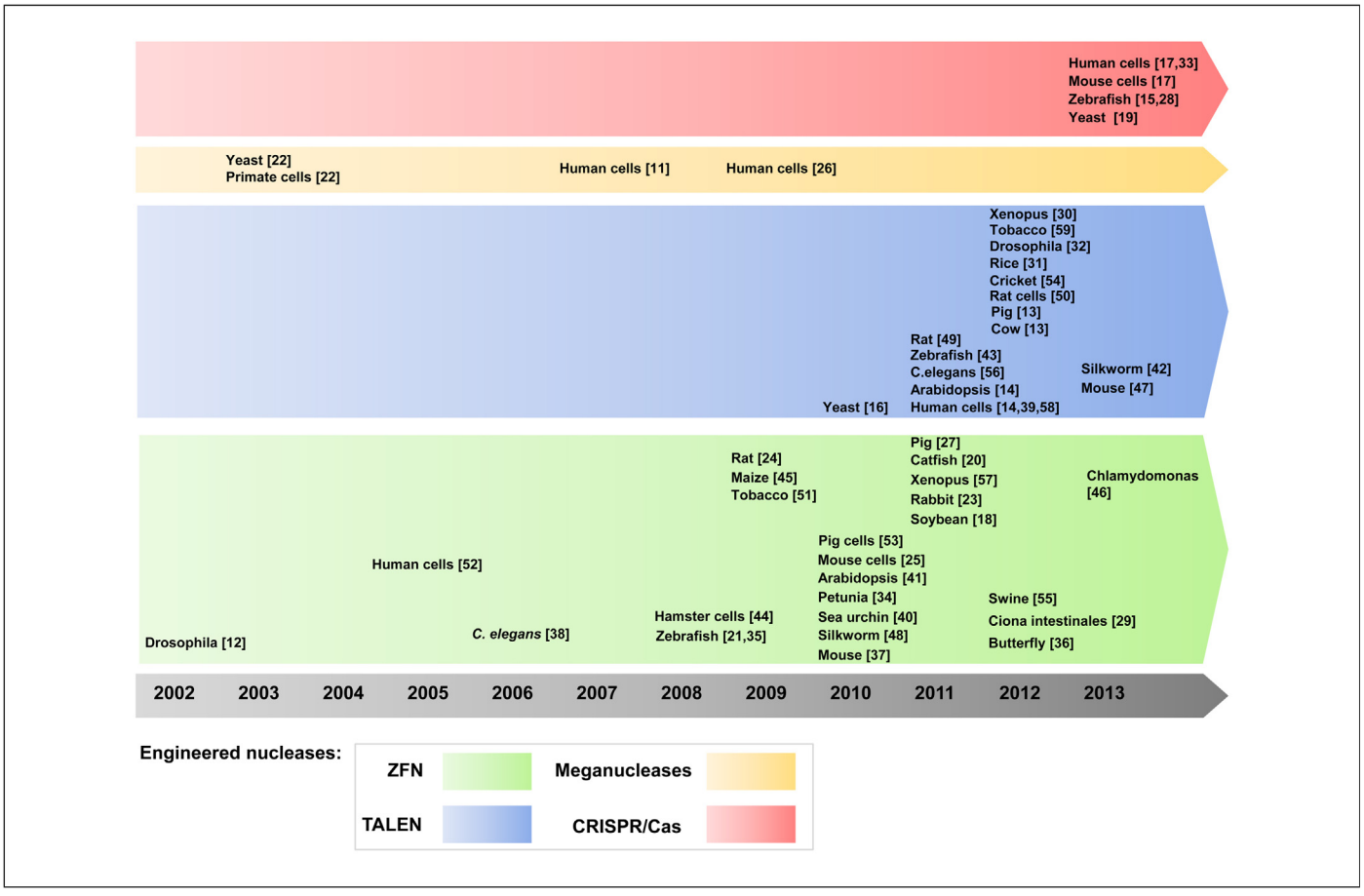

FIGURE 1

Timeline of cells and organisms genetically modified using engineered nucleases. Organisms and/or cells are shown with specific reference to the articles.

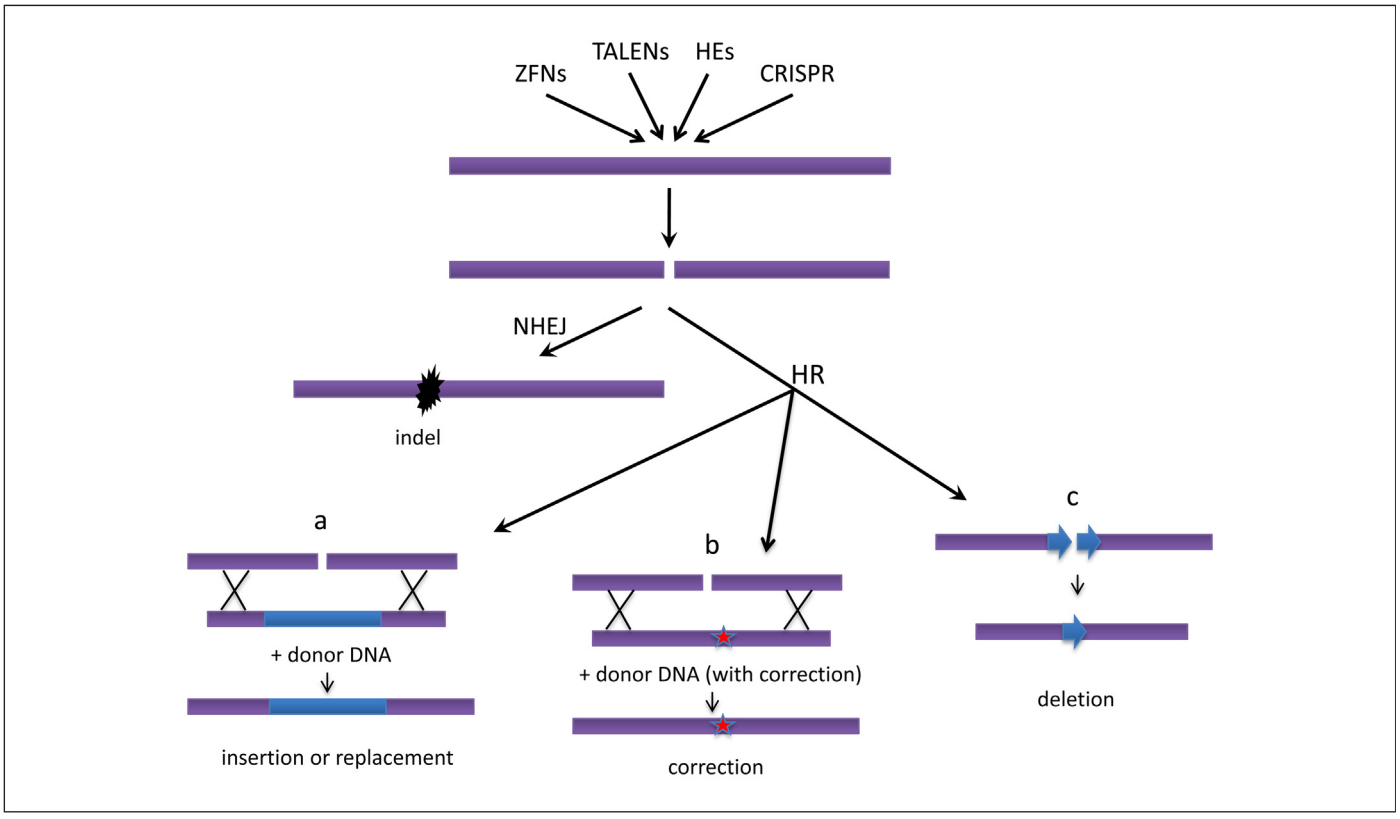

FIGURE 2

Consequences of targeted genomic cleavage. A double-strand break made by targetable nucleases such as zinc-finger nucleases (ZFNs), transcription activator–like effector nucleases (TALENs), meganucleases or homing endonucleases (HEs), or the new CRISPR reagents, can be repaired by error-prone non homologous end joining (NHEJ), leaving small insertions and/or deletions at the site (indel), or by homologous recombination (HR), which can use a manipulated donor DNA as a template, resulting in **(a)** replacement or insertion of genomic sequences **(b)** gene correction or **(c)** the deletion of one repeat.

Several engineering approaches have been developed to create custom optimized ZFNs for novel targets by making use of proprietary archives of single and dimeric ZF modules to produce purchasable ZFNs [21] or by simple modular assembly of publicly available ZF modules into arrays [70]. When ZFs were treated as independent modules, incompatibilities at the finger–finger interface (known as context-dependent effects) often decreased or altered their DNA-binding specificity [71]. However significant progress in efficiency has recently been achieved [72,73] and a publicly available approach, called Context-dependent Assembly (CoDA), explicitly accounts for context-dependent effects between adjacent fingers [74]. The latter effects are also decreased by the use of units that span the finger–finger interspace, ensuring compatibility of neighboring recognition helices [75]. More than a decade of experience has culminated in the design of ZFNs that for the first time are being used in clinical trials [76]. Interestingly, a recent systematic study of the effect of array length and sequence recognition on ZFN activity suggested that, when used appropriately, the modular assembly of ZFNs may offer better results than previously thought [77].

TALENs

Despite the increasing experience gained with ZFNs and until most recent findings were reported [77], the targeting capacity (the diversity of sequences that can be recognized) of ZFNs was considered to suffer limitations [78–80]. TALENs appear to be less subject to these constraints due to the greater simplicity of their DNA-binding domains. These latter are composed of a variable number of 34/35 amino acid repeats [81] (Fig. 3), in which adjacent residues at positions 12 and 13 (the 'repeat-variable di-residue' or RVD) specify a single base pair of DNA [82,83]. Though modules used to recognize G also have affinity for A, TALENs benefit from a simple code of recognition–one canonical module for each of the 4 base pairs – which greatly simplifies the customization of specific DNA-binding domains. Like ZFNs, two individual DNA-binding domains are designed to neighboring DNA sequences, and each is linked to the *FokI* cleavage domain. Each half target site is typically in the range of 14–20 bp, with a spacer of 12–19 bp between binding sites. TALENs can be made on an average of 3 TALEN pairs for any given bp [84,85], whereas ZFNs target sites are reported to be found in diverse sequences on an average of every 500 bp for ZFNs engineered by context-dependent assembly method [74,79] or every 52 bp when using an extended modular assembly of ZFs [77]. Therefore TALENs could surpass ZFN in terms of efficiency, specificity and reproducibility, as shown in tobacco [62,81], in zebrafish [86], and in other studies [14,58,87,88].

Meganucleases

Meganucleases are naturally occurring endodeoxyribonucleases recognizing long (>12 bp) DNA sequences. These are classified in five families of which the LAGLIDADG family (named for a conserved peptide motif) is the largest and best characterized one,

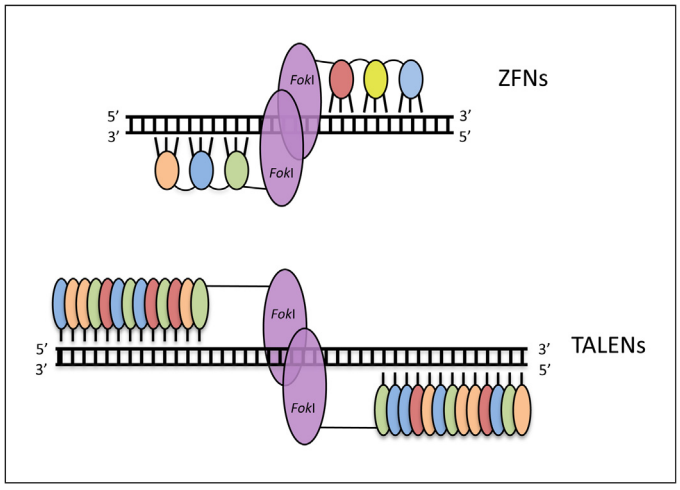

FIGURE 3

ZFNs and TALENs. (Top) Each zinc finger (small ovals) in a zinc-finger nuclease (ZFN) binds primarily to three consecutive base pairs; a minimum of three fingers is required to provide sufficient affinity (up to six fingers have been used per monomer in published studies). Different colors indicate fingers recognizing different DNA triplets. Each set of fingers is joined to a *FokI*-derived cleavage domain (large ovals) by a short linker. The target site is composed of two target halfsites, which are separated by a short optimal spacing of 5–7 nucleotides. ZFN pairs can be designed to bind genomic sequences of 18–36 nucleotides long. (Bottom) In transcription activator–like effector nucleases (TALENs), each module (small ovals) binds a single base pair; the four colors indicate modules for each of the four base pairs. The minimum effective number of modules per monomer is 10–12, but more are typically used. The linker to the *FokI* domain (large ovals) is longer than for ZFNs and contains additional TALE-derived sequences. Image modified from Carroll D. A CRISPR approach to gene targeting. Mol Ther 2012; 20(9): 1658–1660 [62].

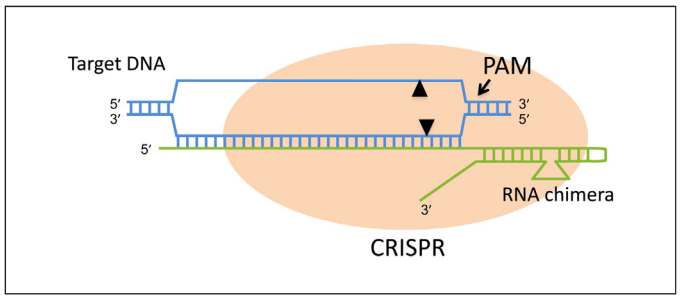

FIGURE 4

The CRISPR minimal-cleavage elements. A single RNA chimera (green lines) with the critical elements of the crRNA and tracrRNA binds Cas9 protein (orange oval) and directs cleavage (arrowheads) to a sequence in DNA (blue) that has homology to the RNA 5′end. The region of RNA–DNA base pairing provides cleavage specificity. The target must also have a particular two base pair sequence adjacent to the region of homology, called PAM, which is recognized by the complex. PAM, protospacer adjacent motif. Image modified from Carroll D. A CRISPR approach to gene targeting. Mol Ther 2012; 20(9): 1658–1660 [62].

and includes the I-CreI and the I-SceI endonucleases. Template based I-SceI-mediated recombination has been successfully applied to induce mutagenesis, recombination between repeats or gene targeting in bacteria [89–92], mosquito [93], fly [94], plants [95–98] and mammalian cells [99] and to improve transgenesis efficiencies in frog [100], fly [101,102] and fish [103]. However, the limited repertoire of meganucleases and hence the limited versatility in terms of targetability is a drawback for genome editing. To allow targeting to other sequences, several meganucleases have been re-engineered. The redesign of the DNA recognition domain is difficult compared to ZFNs and TALENs, since it is not modular; however meganucleases with novel DNA-target specificities have been obtained [11,22,26,104–106]. For example, the redesign of I-CreI endonuclease for genetic modification of plants was first demonstrated in maize by means of targeted mutagenesis of the liguleless locus [107] and homodimeric I-CreI endonuclease has been engineered to target the human RAG1 gene, in which mutations produce severe combined immunodeficiency [108].

CRISPR/Cas

Very recently a prokaryotic RNA-programmable nuclease system has been shown to offer another targeting approach. It relies on the type II CRISPR/Cas system, a type of bacterial immunity that uses RNA–DNA base pairing and a single bifunctional nuclease protein (Cas9) to inactivate invading viral or plasmid DNAs. Expression of tracrRNA (trans-activated CRISPR RNA), pre-crRNA, host RNase III and Cas9 nuclease were shown to be necessary and sufficient for efficient and specific cleavage of DNA *in vitro* [109,110] and in prokaryotic cells [111–113]. Aiming at the direct cleavage of arbitrarily chosen sequences, the RNA portion of this system has been simplified into a single guide transcript, a chimeric RNA fusion of the crRNA3′ end with the tracrRNA 5′ end enabling Cas9 binding and DNA site recognition [110] (Fig. 4). Most recently, the type II CRISPR locus of *Streptococcus pyogenes* has been reconstituted to achieve targeted cleavage in yeast [19], zebrafish [28] and human and mouse cells, including HR in some cases [17,33,114,115]. The type II CRISPR/Cas system has the advantage that recognition is based on base pairing interactions and Watson–Crick complementarity and that it only involves one constant protein. Moreover, the architecture of natural CRISPR loci with multiple tandem guide templates suggests the possibility of multiplexed genome engineering, thereby addressing many targets in a single experiment [17,116].

Nickases

Repair of nuclease-induced DSBs may occur by error-prone NHEJ mechanisms rather than HR, thereby potentially introducing mutations or even translocations at the cleavage site. To avoid such effects, several groups have inactivated one of the nuclease active sites to convert meganucleases [117,118], ZFNs [119] and most recently also Cas9 [17] into nickases that make only single-strand breaks (SSBs). Another approach consisted in fusing the nicking activity of the natural occurring DNA mismatch repair endonuclease MutH with the DNA-binding domain of meganucleases or TALE protein [120]. Since SSBs are not substrates for NHEJ but are repaired either by seamless ligation or high-fidelity HR, the ratio HR:NHEJ is improved and off-target effects are reduced. A 70-fold increase in this ratio was found for meganuclease-based gene correction initiated by SSBs compared to those initiated by DSB [117]. The design and use of ZF nickases was also shown to generate SSBs *in vitro* and to induce targeted HR in cultured human cells with significantly lower rates of associated NHEJ-mediated mutation at the nicking site [119,121]. The drawback to this approach is that the absolute frequency of

cleavage by nickases is significantly lower than that of the DSB versions.

Elements for further improvement

The engineering of nucleases is often driven by the search for improved versatility, utility, robustness, cost-effectiveness, scalability and targetability. Particularly in view of gene therapy applications, researchers also aim at improving the reliability in terms of DNA recognition, an aspect that contributes to the overall predictability and safety of the approach. In that respect, molecular mechanisms associated with NBGT are further explored.

Deciphering DNA-recognition

The ultimate aim would be to allow targeting of all possible genomic sequences by the customization of nucleases which could discriminate in favor of the chosen target and against related sequences in the genome. Customization of ZFNs was considered the most mature approach for several years. However, ZFNs suffer from context effects on DNA recognition and a limited range of targetable sequences, due to the absence of fingers for all possible DNA triplets. The limitations of customization of meganucleases appear even more severe although they are often very specific. TALENs have largely solved these problems with one well-defined module for each of the 4 base pairs. However, some degree of ambiguity has also emerged since the module commonly used to recognize G also binds A. In view of increasing specificity of these G recognizing modules, it was shown that a module with a repeat-variable diresidue Asn-Lys (NK) sacrifices affinity to gain specificity, while modules with an Asn-His (NH) diresidue show improved biological activity and specificity [122]. The CRISPR/Cas system promises to be most flexible in target recognition since it is based on Watson–Crick complementarity. More experimental data are required to see how many mismatches might be tolerated in a cellular context for this system [17].

Off-target activity

Sequence-specificity of engineered nucleases is not absolute and cleavage can occur at sites similar to but different from the target site. Since this could possibly result in unintended mutations or translocations, efforts have been invested to predict and reduce off-target activity. Off-target activity can be caused by low DNA-binding specificity hampering the ability to discriminate between the genuine target sequence and other similar sequences in the genome. This has been observed with the first ZFNs [12] and repeatedly since then [123]. In this regard, results of a broad *in vitro* selection method for active ZFNs indicated that excess DNA-binding energy results in increased off-target ZFN cleavage activity and suggest that ZFN specificity can be enhanced by designing ZFNs with decreased binding affinity, by lowering ZFN expression and by choosing target sites that differ by at least three base pairs from their closest sequence relatives in the genome [124].

Most approaches start with an *in vitro* determination of the consensus DNA binding site for a given pair of ZFN monomers and an *in silico* identification of genomic targets with best homology. In the cell, DSBs are normally rapidly repaired, in some cases leaving no marks or indels, which means that there is a limited insight in the genome-wide identification of all possible off-target ZFN cleavage events and the *in vivo* selectivity of a ZFN in the context of the entire genome. Hence, most of the DNA-recognition design tools still need to be extensively tested and compared in various organisms to assess their predictive power. Interestingly, a genome-wide approach for trapping ZFN induced off-target sites *in vivo* has been developed by the use of integrase-defective lentiviral vectors, which were shown to be captured and ligated into DSBs by NHEJ [125]. A similar approach was used to identify off-target sites of I-SceI meganucleases by using adeno-associated viral vectors [126]. Methods for quantifying ZFN-induced DSB repair foci (genotoxicity assay) and assessing cell survival after application of ZFNs (cytotoxicity assay) have also been described [127].

In view of reducing off-target effects for TALENs, results also pointed to the need to take into account the degeneracy in the TALE-DNA recognition code [83,128]. Tools for optimal TALEN design are continuously being improved [14,84,85,129–131]. It will be interesting to see whether methods for genome-wide trapping of off-target cleavage previously described for ZFN could also trace off-target effects with TALENs.

Off-target effects with *Fok*I-based nucleases, such as ZFNs and TALENs, are also related to the dimerization of the *Fok*I protein needed to achieve cleavage activity. Since undesirable effects, such as cell toxicity, can be due to homodimerization, complementary modifications at the dimer interface of either monomer have been introduced such that heterodimerization is required for cleavage [132–134]. These modifications have led to sharp reductions in off-target cleavage and have reduced observed toxicity in several systems.

Studies where side-by-side comparisons are made between different nuclease scaffolds at defined endogenous target loci are rather scarce, notwithstanding that such studies can provide important information when it comes to the choice and the engineering of the most efficient and safe nucleases. A comparative study between TALENs and ZFNs at two endogenous human loci indicated that TALENs could be as effective as ZFNs in terms of genome modification activity but are significantly less cytotoxic [39]. Another study indicated that TALENs induced indels at significantly higher rates compared to ZFN in Zebrafish [135] and a comprehensive analysis of the frequency of insertions and deletions obtained with ZFNs and TALENs revealed a differential mutation signature independent of the target loci since ZFNs induced insertions much more frequently than TALENs [136]. With regard the single-site specificity of CRISPR/Cas reagents, little information is currently available and more studies are necessary to evaluate the off-target activity in a genomic context [137].

DNA repair mechanisms

A good knowledge of cell specific DSB repair mechanisms could improve the versatility of NBGT, since the outcome of targeted cleavage, regardless of the nuclease, is partly determined by cellular pathways for DNA repair which vary among cell types and developmental stages. The major pathways for DSB repair are HR and NHEJ. NHEJ is an error-prone process that often results in indel mutations. When it comes to gene replacement, the introduction of error-free mutations or the introduction of foreign genes by the use of donor DNA, the outcome will depend on the presence of cellular mechanisms of HR since HR enables a faithful repair at the junction sites upon DSB. In many fungi, HR dominates when a

homologous template is available. In most higher eukaryotes however, NHEJ is the dominant pathway, estimated to occur at a ratio of approximately 1000:1 or more compared to HR [138,139]. Directing repair toward HR by introducing an homologous template can be challenging and has hampered previous attempts for ZFN- or TALEN-mediated gene replacement despite high efficiencies of cleavage [21,35,140]. Recently, a usable level of HR has been reported in zebrafish embryos, using oligonucleotide or plasmid donors [141,142]. As previously mentioned, nickase-mediated SSBs can increase the HR:NHEJ ratio. In Drosophila, it is also possible to switch the balance in favor of HR by inactivating DNA ligase IV, a critical component of the major NHEJ pathway [143,144]. Application of this approach to other systems may be possible, although deleting ligase IV is lethal in some organisms, and alternative NHEJ pathways can compensate to some extent. Another approach to obtaining targeted integrations is to provide a donor with 5′ overhangs complementary to those created by nuclease cleavage and to rely on NHEJ to make the links [145]. The efficiency of this process may be limiting, and events other than the intended one will produce other junctions at the target. Interestingly, another NHEJ-mediated strategy for site-specific gene insertion does not require previous knowledge of the overhangs created upon cleavage and also eliminates the necessity of having homology arms in the donor DNA [146].

Epigenetic mechanisms

Variation in genomic accessibility due to chromatin structure is another aspect that could have an impact on recognition of cleavage sites and efficacy of nucleases. This has been illustrated by several findings: HR-directed gene introduction can be stimulated upon silencing of a gene encoding a protein involved in chromatin remodeling [147]; meganuclease-induced targeting frequency was found to be substantially improved upon treatment of cells with compounds that loosen chromatin structure [147]; TALEN activity can be inhibited by the presence of 5-methylcytosine in targeting DNA and increased by demethylation in human and rodent cells [148,149], and an *in vivo* analysis showed a negative correlation between the rate of indel induction by TALEN and the number of CpG repeats in TALEN target sites [135].

When considering the impact of chromatin structure, however, it is noted that in most, but not all, applications of NBGT, cells are progressing through the cell cycle. During S phase, chromatin structure is disrupted to some extent across the entire genome [150] and it may be in this context that cleavage occurs. The DNA-recognition modules of both ZFNs and TALENs are also derived from transcription factors that bind their targets in chromatin, suggesting that the hybrid proteins may also have the capacity to overcome all but the most severe packaging constraints.

Other considerations not exclusively related to NBGT but caused by alterations in the dynamic interplay between chromatin, small RNAs, regulatory enzymes and DNA sequence include the effect of the introduction of the transgene on the surrounding chromatin structure (*cis* epi-effects), the expression of endogenous genes (*trans* epi-effects) and the effect of epigenetic mechanisms on the expression of the transgene. Further studies and more knowledge on these aspects might give the possibility to better predict the outcome of these effects and to target or avoid specific genomic regions accordingly. However, the need to control these aspects is not equally important for all applications. For GM plants, off-target effects, *cis* and *trans* epi-effects are tolerable as rigorous selection and subsequent breeding programs (similar to traditional plant breeding techniques) ensure that GM plants with undesirable compositional, agronomic or phenotypic features will simply not be retained for further selection. In cases where breeding steps are much more laborious and expensive, such as for GM animal breeding, it is understandable that approaches that minimize selection steps and undesirable consequences gain importance, hence outweighing the benefit of assessing potential alterations in the epigenetic interplay with nucleases. In the context of therapeutic applications, the recipient genome is that of a patient emphasizing the importance of implementing all optimization steps that could reduce potentially harmful unintended effects.

Methods of delivery

While the choice of the method to deliver the nucleases will often be driven by efficacy, specificity and feasibility considerations, the level of expression and the concentration of the nucleases in the cell will also influence the outcome of NBGT. Minimizing off-target effects could be obtained by regulating the amount of proteins so that cleavage is relatively quickly performed and secondary damage is limited [124,151]. Several methods of delivery have been employed. Transient expression of nuclease encoding sequences can be achieved either with DNA-based expression cassettes harboring inducible promoters (*e.g.* by means of *Agrobacterium*-mediated transformation in *Arabidopsis thaliana* [41,152] and/or the use of plasmid vectors [51,153–155] or viral vectors (integrase-deficient lentiviral vectors [127,156], adenoviral vectors [157,158], adeno-associated virus vectors [10,159,160]), which do not integrate into the host genome. Introducing nuclease encoding mRNAs also circumvents the issue of genomic integration and shortens the duration of expression and has been applied to some cells and many model organisms (Drosophila [143], zebrafish [21,35,140], rats [24,161], human iPSCs [162], fertilized oocytes of rabbits [23], sea urchin [40]). In some cases, the nucleases can be introduced directly as proteins [163], but production and purification of adequate amounts often is more challenging. Notably, simultaneous targeted introduction of a repair template and a I-*Sce*I meganuclease was obtained by incorporating the latter as a fusion protein with Vpr in lentiviral particles [164]. Whatever the method of delivery, nucleases can also be endowed with constitutive or inducible degradation sequences to greatly reduce their half-lives in cells [165].

Regulatory oversight of NBGT

The regulation of genetically modified organisms (GMO) includes a safety assessment with respect to their possible impact on human/animal health and environment. Regulatory frameworks have been adopted worldwide to cover new techniques of genetic modification and GMO. Whether or not these frameworks might cover NBTG techniques as well is a consideration that will certainly influence the development and/or commercialization of new products developed with these techniques [166–168]. In the European Union (EU), the ZFN technology has been one of the techniques evaluated in the context of the GMO definition and the provisions of Directives 2001/18/EC and 2009/41/EC

[169,170]. One of the conclusions, which is extrapolatable to other nucleases, was that the use of ZFN to insert foreign nucleic acid sequences should be regulated within the frame of the EU GMO legislation since the latter covers techniques enabling the transfer of genes between organisms that cannot naturally exchange genetic material. Consideration was also given to the use of NBGT to generate indel mutations (error-prone DNA repair by NHEJ) or precise point mutation (using a homologous repair template) resulting in organisms that are similar to, and indistinguishable at the molecular level from organisms obtained through conventional (chemical and physical) mutagenesis methods. From a regulatory perspective, conventional mutagenesis, which induce random mutations in the genome, result in GMOs that are excluded from the EU GMO legislation. Therefore it has been questioned whether disparities in regulation and risk assessment for these products with equal potential to cause harm would be acceptable or desirable [171]. Notably, when evaluating the regulatory and safety issues associated with organisms developed through oligonucleotide-mediated mutagenesis, Breyer *et al.* concluded that a same regulatory status should be applied as for conventional mutagenesis methods [172]. Another regulatory aspect considers whether or not foreign nucleic acids are introduced to the recipient organism in a transient or stable manner. The way nucleases are introduced in the cell and whether or not these are accompanied with repair templates will have an impact on the regulatory status of the resulting organisms in Europe: methods that do not deliver nucleic acids into the recipient cell and that rely on the direct delivery of nuclease proteins without the use of repair templates could be excluded from the EU GMO regulations, whereas the (viral) vector mediated delivery of nuclease encoding sequences, with or without repair templates, might generate GMOs that are regulated.

Within the GMO regulatory framework, the safety assessment of GMOs involves the identification and molecular characterization of the intended and unintended effects as a result of the genetic modification and includes insights on the genomic locus/loci of the modification, the potential impact on the function of any endogenous gene of known or predictable function (*e.g.* by gene disruption) or the generation of new open reading frames. Based on the current regulatory status of conventional mutagenesis methods, such safety assessment might indeed not be suitable or acceptable for organisms wherein indels or point mutations have been introduced by NBGT. With regard to techniques of genetic modification that introduce foreign genetic material and fall into the scope of the GMO regulation, the targeted approach of NBGT offers the possibility to minimize the probability of position effects, and unintended and unwanted gene disruption. In this respect lesser amounts of event specific data may be needed for the molecular characterization and safety assessment of organisms developed by this approach [173,174]. It is also noticed that for biotechnology-based plant breeding applications, screening and selection programs provide a way to intercept potential unintended effects. For NBGT applications where the enrollment of the recipient organisms in such elaborate screening and selection programs is less feasible or even not acceptable (*e.g.* gene therapy), the identification and molecular characterization of intended and potential off-target cleavage sites remain important aspects for the assessment and anticipation of potential unintended and adverse effects.

Conclusion

A majority of GMOs generated so far for basic and applied research, biotechnology, agricultural or medical purposes have been developed by random introduction of DNA into the genome. Besides additional concerns associated with the co-introduction of selectable markers such as antibiotic resistance marker genes, this random introduction could result in unintended effects. NBGT offers the possibility to perform precise editing, replacement or insertion of genes without the use of any selectable markers and to specifically select the targeted genomic region. These features minimize the probability of undesirable random gene disruption, thereby providing a tool for genetic modification that is inherently more predictable and potentially less prone to position effects. NBGT could also provide a way to accelerate the selection and breeding processes for economically important organisms such as crops or livestock. Whether it will have a wider implementation in commercial applications will also depend on the legal status and potential regulatory costs associated with NBGT approaches or products.

NBGT offers a reliable tool for the targeted genome editing applicable to many organisms and much is expected from the further improvement of the technology in terms of efficacy and feasibility. However, the occurrence of unintended effects associated to the use of NBGT cannot be ruled out. For applications where rigorous selection and/or breeding processes are less or not feasible, molecular characterization data can be used to assess the impact of potential off-target and epigenetic effects *in vivo* associated with NBGT.

Author contribution

Persons designated as authors qualify for authorship.

Conflict of interest

The authors declare that they have no conflict of interest. DC receives royalty payments from Sangamo Biosciences for a patent related to the technologies described.

Acknowledgements

The authors thank Fanny Collard, Adinda De Schrijver, and Martine Goossens (Scientific Institute of Public Health, Brussels, Belgium) for their useful contribution to this document.

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