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Gene Editing

Principles and Applications

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PREFACE

Organic evolution is fuelled by the genetic variation generated by spontaneous mutations that are fashioned by the forces of natural selection into adaptations and diversification of bioogical entities leading to progressive changes in their various characteristics. Mutations are extremely useful for both basic and applied researches so that researchers have been in the constant search of tools and techniques for generating precise and predecided changes in the structures and/or functions of genes relevant to their objectives. This quest has resulted in the development of a suite of techniques ranging from chemical and physical mutagenesis, transgenic technology, *in vitro* site-directed mutagenesis, RNAi, antisense RNA and gene disruption technologies. These technologies have been very useful, but each of them offers some advantages and suffers from certain important limitations. For example, the transgenic technology enables utilization of any gene from the entire biological world so that the range, significance as well as novelty of its exploits are staggering. But the transgene integration is generally random and may lead to unpredictable consequences, which necessitates extensive evaluation for performance and biosafety assays. The regulatory approval of transgenic products is demanding and expensive, and their consumer acceptance is lukewarm. Similarly, the gene disruption technique has been used to create many extremely useful gene knockout animal models of human diseases. However, this technology is technically very demanding, involves sophisticated selection procedures, requires genuine ES cells, and is rather expensive. In addition, the frequency of gene integration by homologous recombination is very low.

It was observed during 1990s that the frequency of homology-directed gene integration can be greatly improved by deliberate induction of DSBs. These insights stimulated the development of reagents capable of cleavage at targeted genomic sites, which led to the creation of several site-directed nucleases like ZFNs, TALENs and CRISPR-Cas9. The CRISPR-Cas system is the easiest and the cheapest to redesign and implement for new target sites and is the most widely used. These SDNs, especially the CRISPR-Cas9, have revolutionized biological research since they allow precise targeting of desired genomic sites and introduce all kinds of genetic changes the researchers would be interested in. The various types of genetic changes the researchers would like to induce may be classified into the following three broad groups: gene inactivation, mutation correction, and (iii) mutation induction leading to novel gene functions. Many genes may not be desirable or the researcher may be interested to analyse the

functions of specific genes; in such cases, it would be desirable to specifically and reliably inactivate the desired genes. Many gene mutations lead to undesirable consequences, *e.g.*, human diseases; in such situations, it will be extremely useful to correct the changes in the mutant genes to restore their normal functions. In many other situations, it would be advantageous to modify an endogenous gene to enable it to perform a more useful function or to introduce a new gene into the genome of an organism to generate a useful novel phenotype that is not achievable with the endogenous genes.

SDNs induce DSBs at the targeted genomic sites and the genetic changes are produced during DSB repair by the normal cellular DNA repair pathways. These changes lead to gene inactivation due to indel formation due to nonhomologous end-joining. The correction of mutations in endogenous genes is achieved by sequence replacement due to homology-directed repair. This process can also lead to induction of the desired mutation in an endogenous gene or even integration of complete gene constructs at the specified genomic sites. Sequence replacement for mutation correction/induction or transgene integration requires a suitable donor DNA during exposure of the cells to the selected SDN system. In addition, the CRISPR-Cas9 system has been used to develop base editors for targeted chemical modification of C-to-T and A-to-G leading to single base substitutions at the specified genomic sites. It has also been used to develop epigenetic editors for histone modification and DNA methylation to activate or suppress the expressions of targeted genes. It has also been used to create highly potent genetic devices called gene drives, which can propagate themselves and rapidly spread in crossbreeding natural populations. Gene drives can be used to eradicate harmful natural populations, rescue threatened populations or suitably alter their genetic makeup. But in the absence of appropriate guidelines, gene drives are yet to be field-tested. Finally, effective mechanisms need to be developed for preventing the misuse of gene editing and gene drive technologies by extremist groups.

Genome editing is being used to improve crop plants and other food sources, including animal species, and some of the products have started to reach the consumers. Therefore, policies and procedures for specific regulation of gene-edited plants and animals should be developed and the regulatory laws/regulations of different countries need to be harmonized. Genome editing has been used to generate large animal models of human diseases that are useful for studies on human diseases, screening of drug, etc. Some of the gene-edited animals may possibly serve as donors of organs for humans. Human somatic cell genome editing has been used to develop some gene therapies that are already in clinical use. Human germline gene editing for clinical use is considered 'irresponsible' at present, but CRISPR-babies have already been borne in China. Thus, there is a need for an efficient and effective mechanisms for oversight and governance to prevent inappropriate use of the gene editing technology. The WHO of Unite Nations has constituted a committee that intends to develop a

strong international regulatory framework for germline gene editing and 'a central registry on human genome editing research.'

The gene editing technology is a powerful tool that can be put to many extremely valuable uses, but also opens the door to unexpected and unprecedented perils. For example, a gene drive targeting a female fertility gene is being field-tested by a British firm in Brazil for eradication of *Aedes aegypti* mosquitoes. This application has important benefits for human health, but gene drives can be designed to eliminate even useful species from the face of earth, which may have adverse effects on the ecosystem. Therefore, effective measures must be devised to prevent the misuse of gene editing in general and gene drives in particular by extremist groups.

The present book, Gene Editing: Principles and Applications, attempts to bring to readers the various developments in this rapidly evolving field in a concise, readable and easily comprehendible narrative. The book targets biologists in general and geneticists, biomedical researchers and plant breeders in particular. It is hoped that it will be useful to post-graduate students, research scholars and research workers concerned with analyses of biological phenomena and development of strains with novel and useful traits. The development and various features of chimeric oligonucleotides and the four major SDN systems, *viz.,* ZFNs, TALENs and CRISPR-Cas9, are described in four chapters, one chapter deals with base editors, five chapters discuss the applications of these novel technologies in plants, animals, human health, gene regulation and natural populations and the last chapter discusses the ethical issues and regulations pertaining to the gene editing products.

The authors appreciate the sincere efforts of Mr. Tanay Sharma and his production team, including reviewers, editors, graphics designers and others of Scientific Publishers, Jodhpur for their sincere and commendable efforts that have culminated in the speedy publication of this book in an excellent and attractive form.

In the end, we would like to express our sincere appreciation for the continued affectionate support from all our family members during our involvement in this project. This has been the prime source of our strength throughout our academic life particularly when there were activities like this that demanded the bulk of our time and attention.

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CONTENTS

1

DIRECTED MUTAGENESIS

1.1. INTRODUCTION

The term mutation was first used by DeVries in 1900 to describe sudden changes in the characteristics of *Oenothera* sp. A simple definition of mutation would be a sudden heritable change in the characteristics of an organism. The basis of a mutational event may be a change in chromosome structure or even number, an altered base sequence of a gene/plasmagene or an epigenetic change such DNA methylation. Mutations occur spontaneously at low rates mainly due to errors during replication by DNA polymerase, activity of transposable elements, spontaneous deamination of 5-methylcytosine/cytosine, etc. The rates of spontaneous mutations vary depending on the organism, the gene in question, and several other factors. In general, mutant alleles produce deleterious effects and many incapacitating human diseases are caused by single mutant genes. Significantly, *mutations are the ultimate source of all genetic variation present in the biological world, and genetic variation is essential for organic evolution to proceed*. Thus, the genetic variation utilized for animal and crop improvement has originated from past mutations. There are several instances of spontaneous mutations being utilized for the establishment of new animal breeds/crop varieties. For example, the spontaneous semi-dwarf or reduced height mutants of wheat and rice provided the genetic foundation for the dramatic increase in global cereal production described by many as 'green revolution' (Singh, 2018, 2019).

Mutations are extremely important for a variety of biological investigations, including genetic analyses of phenotypes and dissection of biochemical pathways into individual reactions. However, spontaneous mutations are random, very low frequency (10^{-5} or lower per gene per generation) events. Therefore, researchers have long been trying to develop tools and techniques for inducing mutations in high frequencies and preferably in the desired genes. These persistent endeavours have been amply rewarded beginning with the discovery of physical and chemical mutagens (Section 1.2) and ending with the development of highly specific and efficient site-directed gene/genome editing tools that enable the desired modification in the targeted genes (Section 1.7).

The various types of genetic changes the researchers would like to induce may be classified into the following three broad groups: (i) gene inactivation, (ii)

mutation correction, and (iii) mutation induction leading to novel gene functions. Many of the genes present in genomes of various organisms may not be desirable from one or the other perspective. In addition, the researcher may be interested to investigate the effects of inactivation of specific genes on phenotypes of the concerned organisms. In these and some other situations, it would be desirable to inactivate the relevant genes in a specific manner; several approaches can be used for achieving this objective (Sections 1.5-1.7). But when gene mutations lead to some undesirable consequences, *e.g*., human diseases, it will be extremely useful to correct the changes in such mutant genes to restore their normal functions. This could be done either by providing a new normal copy of the concerned gene or a directed sequence replacement in the mutant gene (Sections 1.8.2, 1.8.3). In many situations, it would be advantageous to introduce a novel gene function into an organism to generate a useful phenotype that is not achievable with the endogenous genes of the species. In such cases, new genes are identified, isolated and cloned, and ultimately transferred and expressed into the concerned species using a suitable gene transfer technology (Section 1.3).

1.2. PHYSICAL AND CHEMICAL MUTAGENESIS

The first notable success at deliberate induction of mutations was reported by Muller in 1927; he used X-ray treatment to induce mutations in *Drosophila.* One year later, in 1928, Stadler demonstrated the mutagenic action of X-rays in barley. These studies provided a tool for induction of heritable changes in genes and raised the possibility of controlled genetic manipulations in organisms. The significance of this discovery is reflected by the award of Nobel prize to Muller in 1946. These research findings stimulated the search for other physical and chemical agents capable of inducing mutations. As a result, several radiations including ultraviolet (UV) rays, and many chemicals like alkylating agents, base analogues, acridine dyes, nitrous acid, sodium azide, etc. were found to induce mutations. The physical and chemical mutagens induce mutations at frequencies that are several orders of magnitude higher than those of spontaneous mutations (Sturtevant, 1965).

The various mutagens have been used to induce mutations in many organisms and the recovered mutants have been extensively used for both basic and applied researches. For example, UV-induced mutants of *Neurospors crassa* were used for studies that led to formulation of the famous one-gene-one-enzyme hypothesis by Beadle and Tatum (1941). Mutagens have been widely used for improvement of agricultural and horticultural crops. As a result, 3,281 mutants belonging to over 200 different crop species have been released as varieties for commercial cultivation (IAEA Vienna Mutant Variety Database, https://mvd.iaea.org/ as on 30.10.2018). Mutagenesis has been successful in the induction of some useful new alleles that were not available/accessible to breeders. It has been particularly useful in correction of specific defects of otherwise well-adapted, high yielding and highly successful varieties. For example, peppermint (*Mentha piperita*) cultivation in U.S.A. is a multimillion-