Genome Editing and Engineering
From TALENs, ZFNs and CRISPRs to Molecular Surgery

Recent advances in genome editing techniques using endonucleases, such as TALENs and CRISPRs, combined with genomic engineering technologies have opened up a wide range of opportunities from applications in basic disease biology research to potential new diagnostic tools and clinical applications.

This complete guide to endonuclease-based genomic engineering gives readers a thorough understanding of this rapidly expanding field. Chapters cover the discovery, basic science, and application of these techniques, focusing particularly on their potential application to the treatment of cancer, and cardiovascular and immunological disease. The final section discusses the legal and ethical issues that accompany the technology. Providing authoritative coverage of the potential that genome editing and engineering carry, this is an ideal reference for researchers and graduate students and those working in the biotechnology and pharmaceutical industries, as well as in a clinical setting.

Krishnarao Appasani is the Founder and Chief Executive Officer of GeneExpression Systems. He is an award-winning scientist, and has edited several books, including: *Optogenetics: From Neuronal Function to Mapping and Disease Biology* (2017), *Genome-Wide Association Studies: From Polymorphism to Personalized Medicine* (2016), *Epigenomics: From Chromatin Biology to Therapeutics* (2012), *MicroRNAs: From Basic Science to Disease Biology* (2007) and *RNA Interference: From Basic Science to Drug Development* (2005).

Genome Editing and Engineering

From TALENs, ZFNs and CRISPRs to Molecular Surgery

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More Information

Dedicated to

Phillip A. Sharp (1944–) 1993 Nobel Laureate Medicine or Physiology and Institute Professor at the Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts.

Phil is an American-born legendary molecular biologist, co-discoverer of split genes and RNA splicing, an entrepreneur and futurist, who foresaw the new field of RNA interference and genome editing.

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Foreword

I am thankful to Krishnarao Appasani for making the extraordinary effort to prepare this timely volume on the rapidly expanding field of genome editing and engineering, and for inviting me to offer a few of my own initial statements.

We live in exponentially exciting times. Times in which we can design and build new versions of industrial microbes, agricultural plants, animals and even ourselves. Our ability to sequence and synthesize DNA has improved over four-millionfold in just a few years, such that trillions of base pairs are now read and written by automated chip-based systems. The cost of editing a single base pair has improved about tenfold in that same time frame. It is important to keep in mind that the revolution is just as much about sequencing and synthesis as it is about editing. Without facile reading of whole genomes, we would lack key tools that permit avoidance of off-target editing. Without the synthesis of DNA on chips of large libraries of barcodes, guide-RNAs or sequence-specific DNA-binding protein domains, we would not be well positioned to conduct large screens for function.

When we speak of genome engineering, this is not just about one type of endonuclease, so it is refreshing to see representation in this book of meganucleases, zinc-finger nucleases, TALENs and CRISPRs. Moreover, genome engineering is not simply about nucleases, but other editing methods, especially those which intrinsically avoid the curse of non-homologous end-joining (NHEJ). These include phage-derived integrases/recombinases (such as lamba–int, cre, Bxb1), MAGE recombineering and adeno-associated virus (AAV) homologous recombination. In addition to increasing the on-target precision, these non-nuclease methods can reduce the toxicity of double-strand DNA breaks and hence increase the multiplicity. To put this in perspective, the record for multiplex edits per genome is 62 for mammalian cells, 25 for a whole living mammal (pig), and 321 for a bacterium (recoded *E. coli*). New efforts have begun, collectively called "Genome Project Write" (GPW), which involve 62 000 edits (*E. coli*) and 400 000 edits (human genome) to make cells resistant to all viruses. This indicates how crucial technology development will be in the near future for editing, screening, testing and debugging of complex functional interactions. So, it is noteworthy

that this text contains over a half dozen chapters on these technology development topics.

This volume covers the full spectrum from model organisms to clinical applications. Organisms include representatives of crustaceans, nematodes, fish, frogs, mice, pigs and humans. Naturally, within experiments on human cells we have additional topics well resourced in this book, including pluripotent stem cells, organoids, transplantation, cancer and regenerative biology, modeling and treating disease biology. Thankfully, in this context the final four chapters cover the very pressing and timely issues of patents, safety and ethical concerns of genome editing.

This comprehensive book is welcome at this point of rapid growth in new technologies and discoveries, such that keeping up with applications of genome engineering is challenging. Here we have both a general introduction to the field and to deep mechanisms and glimpses of future applications and ethical considerations – with gems for newcomers and experts alike. It is a great pleasure to participate in this grand experiment of sharing protocols, reagents and viewpoints on how to proceed responsibly.

March 21, 2018

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Preface

Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world; and in the fields of observation chance favors only the prepared mind.

– *Louis Pasteur*, a French microbiologist (1822–1895) who discovered the principles of microbial fermentation and pasteurization

The famous Austrian British philosopher of science Karl R. Popper (1902–1994) believed that "before we can find the answers, we need the power to ask new questions, in other words, we need new technology." A key example of such a technological advance is the recent development of the new field of genome editing and/or genome engineering developed by several pioneers. Genome editing can be defined as "a powerful new tool for making precise additions, deletions, and alterations to the genome – an organism's complete set of genetic material." The development of efficient and reliable ways to make precise, targeted changes to the genome of living cells has been a long-standing goal for biomedical scientists. To reach this goal, researchers have utilized three different approaches in recent years: (1) zinc-finger nucleases (ZFNs), (2) transcription-activator like effector nucleases (TALENs) and (3) clustered regularly interspaced short palindromic repeats (CRISPRs).

Both ZFN and TALEN technology platforms have enabled researchers to generate permanent mutations by introducing double-stranded breaks to activate repair pathways. These approaches are relatively costly and time-consuming to engineer, limiting their widespread use for large-scale high-throughput studies. Recently, a new tool based on a bacterial CRISPR-associated protein-9 nuclease (Cas9) from *Streptococus pyogenes* has generated considerable excitement. This follows several attempts over the years to manipulate gene function, including homologous recombination and RNA interference, but they were hampered by providing only temporary inhibition of gene function and unpredictable off-target effects. The first section of this book details the biology of these endonucleases and their regulatory networks.

Genome Editing and Engineering: From Talens, ZFNs and CRISPRs to Molecular Surgery is intended for those in the genetic engineering, molecular agriculture,

stem cell biology, biotechnology, genetics, genomics, pharmaco-genomics and molecular medicine fields. There are a few books already available covering genome editing and various endonucleases.[1–7] Most of these books primarily focus on methods and lab protocols, except the recent book by Doudna and Sternberg, which discusses details of the field of genome editing and its bioethical issues and evolutionary perspectives. This present book differs, in that it is the first text completely devoted to combining endonuclease biology and genome editing use in model organisms, technology platforms and applications in studying the pathology of many diseases, as well as stem cell biology. Special emphasis is given to highlight the studies of model organisms, novel screening platforms that have been applied to regenerative and disease biology. This authoritative volume brings together the views of global experts on the use of genome editing in disease research, including cancer, cardiovascular disease, infectious disease and immunity, as well as RNA biology, HIV, retinal diseases and the development of therapeutics. Importantly, this authoritative guide also covers the legal and bioethical issues related to this emerging field of modern biology.

Repeated sequences in *E. coli* were initially discovered by a Japanese group,[8] followed by Francisco Mojica from the University of Alicante, Spain in 1995.[9] In parallel, two French groups have also identified such sequences,[10,11] however its "adaptive immunity function" was demonstrated by Phillippe Horvath and his colleagues.[12] There are many more heroes who have played key roles in the identification of various nucleases, but their potential was realized in 2012 by Jennifer Doudna of the University of California, Berkeley, CA, USA[13] and Emmanuelle Charpentier of the Helmholtz Center for Infection Research, presently at the Max-Planck Institute of Infection Biology, Germany.[14] Later, Feng Zhang's group at MIT, USA also adopted this technique for precise engineering of genomes and developed several reagents, making them public to the research community worldwide.[15] Since then, targeted genome editing has brought about a new revolution in biological research.

Although genome editing arose from microbiology, it addresses a much broader unmet need in the study of biological systems beyond microbiology, in everything from agriculture to embryonic biology. The editing phenomenon has been observed in many model organisms, including bacteria, round worms, flies, fish, mice and humans. The second section of this volume covers genome editing processes in model organisms and the third section focuses on technology development and screening methodologies.

CRISPR/Cas-9 system-related genes are essential in "adaptive immunity" in selected bacteria, the archaea, enabling an organism to respond to and eliminate invading genetic material. The CRISPR/Cas-9 system originates from type II endonucleases, which provide bacteria with adaptive immunity to viruses and plasmids. This is a remarkable technology that can be used to precisely and efficiently target, edit, modify, regulate and mark genomic loci of a wide array of cells and organisms. Genome editing was hailed as the 2011 "Method of the Year" by *Nature Methods*, and in 2015 the CRISPR/Cas9 system was named the

"Breakthrough of the Year" by *Science* magazine. Additionally, a special issue of *Nature* was launched in 2016 with a cover headline reading "CRISPR Everywhere."

Genome engineering with the RNA-guided CRISPR/Cas-9 system in animals and plants is changing the face of biology. It is easier to use and more efficient than other genetic engineering tools, so it is already being applied in several thousand labs all over the world, just a few years after its discovery. The number of scientific publications on this technique also has been skyrocketing. The "CRISPR craze" has spread worldwide and researchers are adopting it for analysis of functional genomics, large-scale screening for drug targets and helping to engineer animal models that will benefit pharmacological studies. These endonucleases (especially CRISPR-Cas9) act like molecular scissors, cutting and replacing DNA letters in an organism's genome with exquisite precision and ease. The technique is revolutionizing the study of species from mice to potatoes, and is likely to open up powerful new avenues in gene therapy for the treatment of human diseases – "genome surgery" is becoming a reality.

Recently genome editing techniques have been used to alter embryos in monkeys and reduce unwanted mutations in the mitochondria in the eggs or embryos of mice. Such applications of genomic editing tools in stem cells and regenerative biology are covered in section four of this volume. Genome editing potentially offers a powerful approach to treat many human diseases, including HIV/AIDS, hemophilia, sickle-cell anemia and several forms of cancer, as we move closer to the clinic. The applications of genome editing and genome engineering techniques have been widely adopted to study disease biology, as detailed in the fifth section of this book. Nearly half a century ago, Bernard Davis, a Harvard microbiologist, envisioned the promise, the risks and the roadmaps for genetic research, including research on making heritable changes in the germline.[16] A note of caution is that genome editing in human embryos using current technologies could have unpredictable effects on future generations, and there are clearly important questions to be answered. The final section of this volume is devoted to cover such bioethical and legal (intellectual property) perspectives. Last year, the "Human Gene-Editing Initiative" was formed in collaboration with the US National Academy of Sciences and the US National Academy of Medicine, by inviting an international panel of scientists to set up a framework and guidelines for the use of genome editing tools in humans, especially relating to assisted reproductive technologies, stem cell therapies, gene transfer and mitochondrial replacement techniques (www.nap.edu/10766). As mentioned by Eric Lander of MIT and Harvard in his seminal review,[17] "the genome-editing discoveries are examples for both 'hypothesis-free' and 'hypothesis-driven' science and the partnership between these two approaches will be seen quite often in this 21st century."

The goal of this book is to serve as a reference for graduate students, postdoctoral researchers and primary investigators, as well as an explanatory analysis for executives and scientists in molecular medicine, molecular engineering, biotechnology and pharmaceutical companies. Our hope is that this volume will serve both as a prologue to the field for newcomers and as a reference for those

already active in the field. Most importantly, this book serves as a bridge between the basic science of CRISPR and its diverse applications in areas such as agriculture and biomedicine.

We have carefully selected the chapters, written by experts in the field from both academia and industry, and have divided the chapters into appropriate sections to support the theme expressed in the subtitle of this book: From TALENs, ZFNs and CRISPRs to Molecular Surgery. Developing novel genome editing tools is likely to become a prerequisite for genome engineering studies used to alter the genetic makeup. The elegant and revolutionary genome editing approaches that are covered in this book will undoubtedly have great future commercial promise for the development of innovative molecular surgical procedures and for the study of biomedicine and human disease biology.

Many people have contributed to making our involvement in this project possible. We thank our teachers for their excellent teaching, guidance and mentorship, which have helped us to bring about this educational enterprise. We are extremely grateful to all of the contributors, without whose commitment this book would not have been possible. Many people have had a hand in the preparation of this book. Each chapter has been passed back and forth between the authors for criticism and revision; hence each chapter represents a joint contribution. We thank our reviewers, who have made the hours spent putting together this volume worthwhile. We are indebted to the staff of Cambridge University Press, and in particular to Katrina Halliday for her generosity and efficiency throughout the editing of this book; she truly understands the urgency and need of this volume. We also extend our appreciations to Noah Tate for their excellent cooperation during the development of this volume. We want to thank Professor George Church, an American-born geneticist from the Harvard University Medical School, USA and one of the pioneers in the field of modern genetics and genome engineering, for his kindness in writing a Foreword to this book. Last, but not least, we thank Shyamala Appasani for her understanding and support during the development of this volume.

This book is the ninth in the series *Gene Expression and Regulation* that we have worked on and the fourth joint project between father and son. A portion of the royalties will be contributed to the Dr. Appasani Foundation (a non-profit organization devoted to bringing social change through the education of youth in developing nations) and The MINDS Foundation (Mental Illness and Neurological Diseases), which is committed to taking a grassroots approach to providing high-quality mental healthcare in rural India.

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