DNA/RNA heteroduplex oligonucleotide for highly efficient gene silencing

Takanori Yokota
Professor,
Department of Neurology and Neurological Science,
Graduate School of Tokyo Medical and Dental University,
TMDU

HETERODUPLEX OLIGONUCLEOTIDE (HDO) is a brand new oligonucleotide drug, pioneered by researchers of Tokyo Medical and Dental University, Osaka University and ISIS. HDO is found to be significantly potent at reducing expression of the target RNA, and also effectively improves the phenotype in disease models. In addition, the high potency of vitamin E-conjugated HDO results in a reduction of liver dysfunction. HDO technology was anticipated as a basic technology of molecular targeted therapy. The results are scheduled for publication online in "Nature Communications" on August 10th.

"Two major types of RNA targeting oligonucleotide drugs are currently being developed as therapeutic platforms for reduction of target gene expression; short interfering RNA (siRNA) and RNase H dependent antisense oligonucleotides (ASO), says corresponding author Takanori Yokota, MD, PhD, professor of department of Neurology and Neurological Science at Tokyo Medical and Dental University.

Like most medical drugs, methods which further increase potency of oligonucleotide drugs and improve safety and tolerability are highly desirable. The insufficient delivery, poor cellular uptake of oligonucleotides and their inefficient access to target RNA are major impediments to in vivo silencing. Here we developed a novel short DNA/RNA heteroduplex oligonucleotide (HDO). HDO has a structure different from double-stranded RNA used for siRNA and single-stranded DNA used for ASO, and different functional molecular mechanisms from siRNA or ASO in the cells. HDO is composed of DNA/locked nucleic acid (LNA) gapmer as ASO and its complementary RNA (cRNA). When α-tocopherol (vitamin E) as drug delivery moiety conjugated to ASO directly, its silencing effect is reduced because conjugated lipid interfers the mechanisms of ASO. On the other hand, when α-tocopherol is conjugated to cRNA of HDO, α-tocopherol can improve delivery of HDO to the liver, making the gapmer DNA strand active by its release from HDO due to cleavage of cRNA by cellular nuclease (figure)"

Toc-HDO is significantly more potent at reducing the target messenger RNA compared to the parent ASO. Effective Dose 50 (ED50), which means the dose required 50% reduction of target gene Toc-HDO targeting Apolipoprotein B (ApoB) mRNA(ED50, 0.038 mg/kg), was 22.2 times more potent than the parent ASO (ED50, 0.841 mg/kg) in the liver. In addition to lowering ApoB mRNA, the Toc-HDO can reduce serum low-density lipoprotein (LDL)-cholesterol with the pharmacological effects lasting more than one month based on a Toc-HDO injection of 0.75 mg/kg without the need for an ASO injection. Furthermore, highly potent the suppression of the target messenger RNA is observed not only in rodents but also non-human primates."

Mipomersen, the first oligonucleotide drug, was approved by FDA, though not by EU due to liver toxicity. The high potency of Toc-HDO results in reduction of liver dysfunction observed in the parent ASO with the same silencing effect is probably due to a smaller administered dose of nucleotide.

These results suggest that DNA/RNA heteroduplex can serve as the basic technology of the oligonucleotide drug, and opens up a new horizon for human gene therapy as a novel class of oligonucleotide drugs. Our team is currently investigating more detailed mechanisms of HDO and application of ligand-conjugated HDO to another organs, including the brain. TMDU developed a brand new bio-bencher company called "RENA therapeutics" for clinical application of the HDO technology.

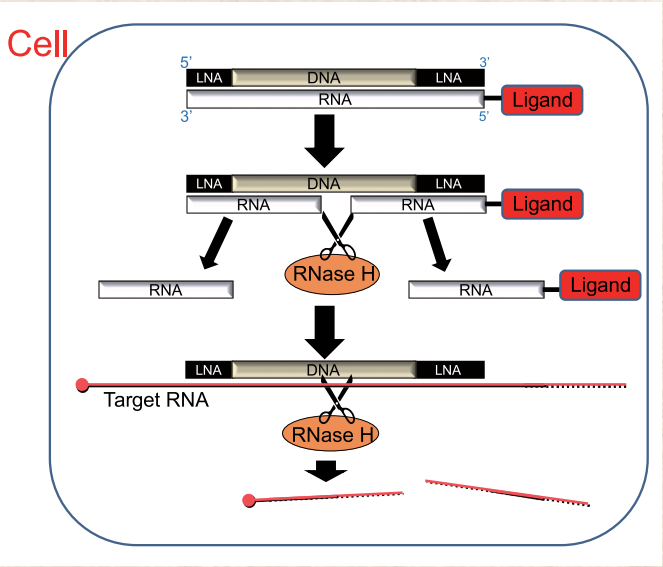

Fig.1: Intracellular mechanism of DNA/RNA heteroduplex oligonucleotide(HDO).
Delivery ligand bound to the complementary RNA strand is cleaved by endogenous RNase H which recognizes DNA/RNA heteroduplex. This cleavage makes the DNA/LNA gapmer strand active.

Discovery of novel gene mutations associated with lethal arrhythmias during exercise

Tetsushi Furukawa
Professor,
MD, PhD,
Bio-informational Pharmacology,
Medical Research Institute,
TMDU

THE HEART HAS changed its shape and function during the course of evolution. Creatures living under water have 2 chambers, one atrium and one ventricle, and electrical signals propagate uni-directionally. In contrast, creatures living on land have 4 chambers, two atriums and two ventricles: electrical signals propagate downward in atriums, while ventricles need to send blood into aortas located above the ventricles, and thus electrical signals have to propagate upward (reverse propagation). To achieve the reverse propagation of electrical signals, ventricles are equiped with a special conduction system called the "His-Purkinje system". The His-Purkinje system is a newly developed system found only in birds and mammals. Various clinical and experimental studies indicate that the His-Purkinje system plays an important role in the development of lethal arrhythmias and sudden deaths. However, it is unknown how the His-Purkinje system links to lethal arrhythmias.

Irx3 is a transcription factor present only in the His-Purkinje system in the heart. Irx3 activates the transcription of Scn5a encoding the cardiac sodium channel and Cx40 encoding a gap junction channel present mainly in the His-Purkinje system. Scn5a and Cx40 are known to be responsible for the fast conduction of the His-Purkinje system. Thus, Irx3 is a key transcription factor determining the fast conduction of the His-Purkinje system. We examined the relationship between Irx3 and lethal arrhythmias in mice and humans.

In Irx3-/- mice, surface electrocardiogram (ECG), and contraction and relaxation of the heart were normal in the baseline. Thus, Irx3-/- mice have apparently normal hearts in the baseline. Continuous ECG monitoring revealed advanced atrio-ventricular (AV) block and frequently occurring non-sustained ventricular tachycardias (VTs), only during the night, an active phase of mice. We challenged exercise stress and a sympathetic nervous system agonist. Both challenges induced the development of AV block and non-sustained VTs. An ex vivo optical mapping during a sympathetic nervous system agonist application revealed the impairment of the reverse propagation of electrical signals and rapid conduction in the ventricles.

Next, we investigated whether IRX3 genetic defects also cause arrhythmias in humans. In 130 patients with idiopathic ventricular fibrillation (VF) and 250 controls, we sequenced exons of IRX3. We found two novel IRX3 mutations in idiopathic VF patients, but none in controls. We also found a common IRX3 variant in three idiopathic VF patients (2.3%) and the same common variant in one control (0.4%). In those five patients with IRX3 mutations or variant, VF occurred related to physical activities. We confirmed in in-vitro experiments that those IRX3 mutations or variant decreased the expressions of SCN5A and CX40.

Sudden death due to VF related to exercise occurs in about one out of 10,000 individuals with normal hearts. Some genetic diseases, such as long QT syndrome and arrhythmogenic right ventricular cardiomyopathy, are implicated in the cause of sudden death related to exercise. Our data adds the genetic dysfunction of IRX3, a transcription factor specifically expressed in the His-Purkinje system, as a possible cause of sudden death related to exercise. The His-Purkinje system is equipped to improve cardiac function during the course of evolution in birds and mammals. Its genetic defects produce exercise-related lethal cardiac arrhythmias as an apparent evolutionary trade-off.

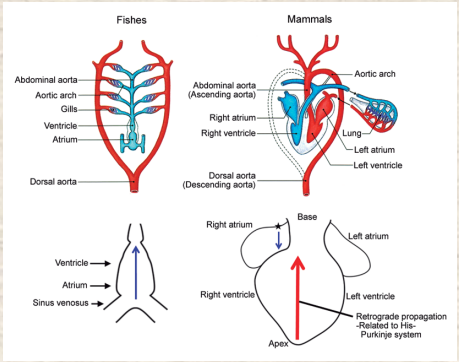

Fig.1: **Evolution of the heart and the ventricular conduction system.**
Upper: Evolution of the heart from fishes with 2 chambers to mammals with 4 chambers.
Lower: Evolution of the cardiac conduction system from fishes with uni-directional propagation to mammals with the retrograde propagation in the ventricle.

References

Koizumi A, Sasano T, Kimura W, Miyamoto Y, Aiba T, et al. Genetic defects in a His-Purkinje system transcription factor, IRX3, cause lethal cardiac arrhythmias. Eur. Heart J. 2015 (in press).

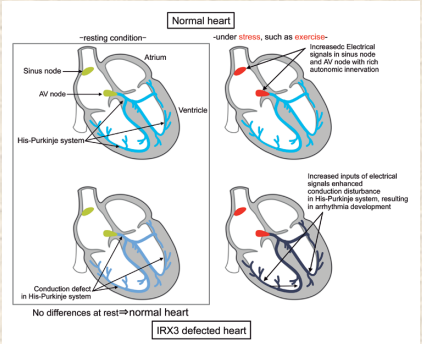

Fig.2: **The possible mechanism of exercise-related arrhythmias by IRX3 genetic defects**
Upper: Normal hearts, Lower: IRX3 defected hearts, Left: Resting condition, Right: Under stress, such as exercise. In the resting condition, the functions of the heart with IRX3 genetic defects cannot be distinguished from that of the normal heart (lower left). Under stress, such as exercise, increased inputs of electrical signals into the His-Purkinje system manifest the conduction disturbance, resulting in the development of arrhythmias.

Cloning-free CRISPR/Cas9 system

Kohichi Tanaka
Professor,
MD, PhD,
Molecular Neuroscience,
Medical Research Institute,
TMDU

THE MOUSE HAS become the most commonly used animal in the biological and medical sciences because its genome can be specifically modified with nucleotide precision. Recent advances in genomic microarray and next generation sequencing technologies have identified many variants associated with common and complex human diseases. To determine whether these variants are causal for human diseases, we need to investigate their biological function. One possible approach is the use of genetic mouse models incorporating the identified variants. However, traditional gene targeting in embryonic stem (ES) cells, although suitable for carrying any desired genetic modifications, is laborious and time-consuming. The development of clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated endonuclease (Cas) is revolutionizing genetic engineering in the mouse. This technology depends on the cell processes triggered by the DNA double strand break (DSB) in specific DNA sequences (Fig. 1). The non-homologous end-joining (NHEJ) pathway, which repairs DNA damage in the absence of template DNA, results in the introduction of random insertions or deletions that disrupt gene function. In contrast to NHEJ, homology-directed repair (HDR) uses a DNA donor template with homology to the DSB site to achieve precise homologous recombination. This method provides exciting and groundbreaking opportunities, enabling direct and rapid gene targeting in fertilized mouse eggs, with no need for ES cells. Using in-vivo genome editing, genetically engineered mice can be created in months rather than years.

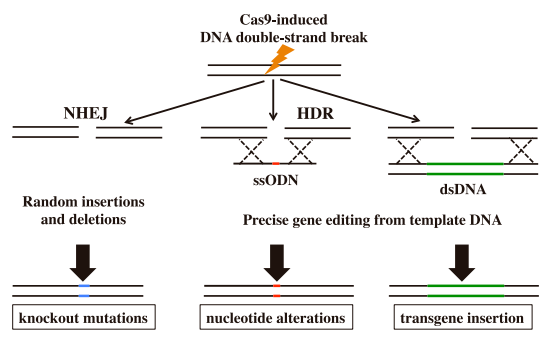

Fig. 1: **Cas9-induced genome editing**
NHEJ, non-homologous end joining; HDR, homology-directed repair; dsDNA, double-strand DNA; ssODN, single-stranded donor oligonucleotides

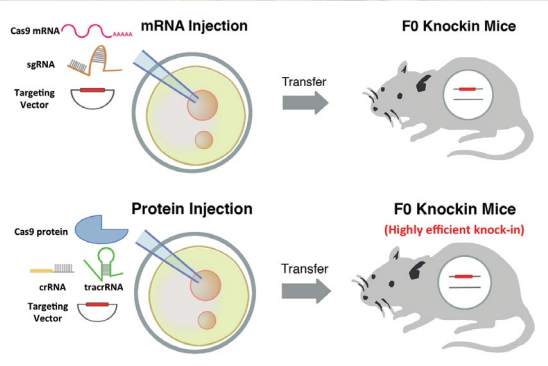

Fig 2: **The standard and cloning-free CRISPR/Cas9 system**
A: The standard CRISPR/Cas9 ststem
B: Cloning-free CRISPR/Cas9 system, which leads to super efficient targeted insertion of a long targeting vector into mouse genome.

A flood of studies using CRISPR/Cas-mediated in-vivo genome editing have reported the production of knock-out mice and knock-in mice carrying single nucleotide substitutions combined with oligo DNA donors. In contrast, there has been only one report on the successful production of knock-in mice carrying reporter gene cassettes, essential tools for analyzing complex tissues such as brain in vivo, and the efficacy of the targeted insertion of the reporter gene was only about 10%. The low success rates of gene cassette knock-in limit the applicability of CRISPR/Cas-mediated in-vivo genome editing.

I have now overcome this issue by developing innovative highly efficient CRISPR/Cas system, which resulted in targeted insertion of long gene cassettes including enhanced green fluorescent protein (EGFP), into the mouse genome of fertilized eggs with an efficiency factor of up to approx. 50% (Genome Biol. 16:87, 2015). I reproduced the natural state of CRISPR/Cas system, which consists of three components—Cas9 protein, chemically synthesized crRNA, and tracrRNA—instead of the commonly used two-component system which consists of Cas9 mRNA and sgRNA, leading to extremely high efficiency (Fig. 2). The cloning-free CRISPR/Cas system further provides highly convenient and accurate gene modification, and its successful transmission to the next generations.

This improved CRISPR/Cas system will be useful for a variety of applications, including creation of humanized mice for modeling of genetic diseases, drug metabolisms, immunity, and infectious diseases. Further, accurate targeted insertion will improve the safety of gene therapy in human patients in the future. Taken together, our streamlined cloning-free CRISPR/Cas-mediated in-vivo genome editing system provides highly efficient and extremely convenient one-step generation of knock-out and knock-in animals, leading to acceleration of in-vivo functional genomic research.

HLA imputation methods and its application to Graves' disease in Japanese

Yukinori Okada
MD, PhD,
Junior Associate Professor, Human Genetics and Disease Diversity, Graduate School of Medical and Dental Sciences, TMDU

GENETIC VARIANTS IN the major histocompatibility complex (MHC) region explain large components of genetic background for various diseases and biomarkers. The MHC region is one of the most polymorphic loci in the human genome, and includes multiple human leukocyte antigen (HLA) genes. While risk fine-mapping of the HLA gene variants contributes to elucidation of genetic architectures of diseases, it has been challenging due to the complex structures of the regions. To this end, we constructed a new population-specific HLA reference panel of Japanese ancestry as a largest dataset ever (n = 908). In the panel, genotype data are available for the three class I HLA genes (HLA-A, HLA-B, and HLA-C) and four class II genes (HLA-DRB1, HLA-DQB1, HLA-DPA1, and HLA-DPB1), as well as single nucleotide polymorphisms (SNPs) densely genotyped using multiple commercial microarrays.

Using our reference panel, we can conduct highly accurate in-silico imputation of the HLA allele genotypes of the pre-existing genome-wide association (GWAS) data. Additionally, we conducted trans-ethnic comparisons of the linkage disequilibrium (LD) and haplotype structures of HLA variants using our Japanese reference panel and previously constructed East Asian and European panels. We applied two novel methods, an entropy-based LD measurement (ε) and a visualization tool to capture high-dimensional variables (Disentangler). The symbol ε represents the normalized entropy difference in the haplotype frequency distributions between LD and linkage equilibrium (LE). Disentangler is a graphical tool designed for visualizing high-dimensional haplotype data across multiallelic genetic markers such as HLA alleles. Our analysis demonstrated population-specific features of HLA allele genotype LDs. In particular, we found that the Japanese population had a relatively stronger LD between HLA genes compared with other populations, which was characterized by one Japanese-specific common long-range HLA haplotype running through the entire MHC region (Fig. 1).

Finally, we applied HLA imputation to a large-scale GWAS data of Graves' disease (GD) in Japanese (n = 9,003).

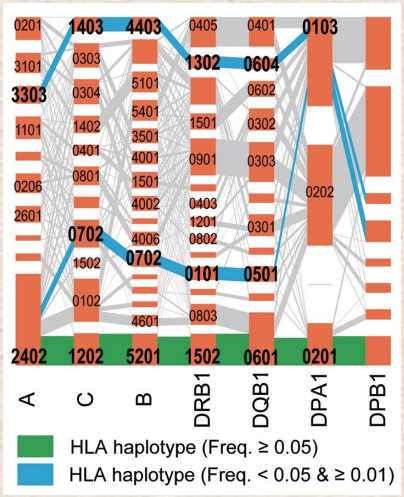

Fig.1: **HLA allele structure of the Japanese population**
Using multiple BIG DATA analyzing methods, we identified that the Japanese population had one Japanese-specific common long-range HLA haplotype spanning the entire MHC region (colored in green).

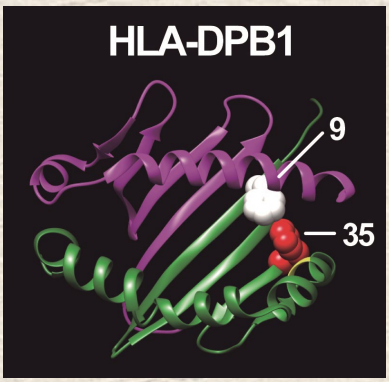

Fig.2: **HLA-DPβ1 amino acid positions with Graves' disease risk**
Application of the HLA imputation method to Japanese Graves' disease GWAS data identified that specific amino acid positions of HLA-DPβ1 confer disease risk.

GD is a disorder of the immune system that results in the overproduction of thyroid hormones, and it affects approximately 2% of the global population. While GD is known as a heritable trait and contribution of the HLA genes to GD genetic risk has been suggested, detailed fine-mapping analysis has not been conducted so far. Comprehensive statistical analysis, including a stepwise conditional analysis and a multivariate regression analysis, identified that amino acid polymorphisms of the multiple class I and class II HLA genes independently contribute to the risk of GD (HLA-DPβ1, HLA-A, HLA-B, and HLA-DRβ1). Of these, the amino acid position 35 of HLA-DPβ1 demonstrated the strongest impact on disease risk (odds ratio = 1.4, P = 1.6×10-42; Fig. 2). It is interesting that independent genetic risk has been identified as both class I and II HLA genes despite their different roles in autoimmunity. Considering strong odds ratios of these identified risk HLA gene polymorphisms, our findings should contribute to disease onset predictions based on personal genome data.

In summary, our analysis clearly illustrates the value of population-specific HLA reference panels for MHC risk fine-mapping. We are now applying our HLA imputation method to additional diseases through internal and international collaborations. The Japanese HLA reference panel is publicly available from the Japanese Genotype-phenotype Archive (URL: https://ddbj.nig.ac.jp/jga/viewer/view/study/JGAS00000000018).