—Review—

Basic and Clinical Studies on Functional RNA Molecules for Advanced Medical Technologies

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Abstract

Small interfering RNAs (siRNAs) and microRNAs (miRNAs) are functional RNA molecules that have recently emerged as important regulators of gene expression at the posttranscriptional or translational level. The RNA interference effects of siRNA on gene expression make it a valuable research tool for knocking down the expression of genes in mammalian cells *in vitro* and *in vivo* enabling the elucidation of molecular mechanisms underlying human diseases. Endogenous miRNAs are involved in a variety of physiological and pathological processes in humans. In this mini-review we first address the synthesis, mechanisms of action, and functions of siRNAs. Then, we focus on recent advances and technologies in miRNA and protein research of the human placenta. Next, we discuss the clinical applications of miRNA in lung cancer. We also touch on "long" noncoding RNAs from intergenic regions of the human genome. This review article is based on a presentation given at a symposium entitled *Basic and Clinical Studies on Functional RNA Molecules for Advanced Medical Technologies* held at Nippon Medical School in Tokyo, Japan, on November 7, 2009.
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Key words: small interfering RNA, microRNA, noncoding RNA, placenta, lung cancer

Introduction

This review article is based on a presentation given at a symposium entitled *Basic and Clinical Studies on Functional RNA Molecules for Advanced Medical Technologies* held at Nippon Medical School in Tokyo, Japan, on November 7, 2009. The aim of the presentation was to describe recent advances in medical technology related to functional RNA molecules and to show how RNA molecules can improve our understanding of pathophysiological

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functions related to human health and disease. Kumiko Ui-Tei presented effective and target-specific gene silencing, regulated by small interfering RNA (siRNA) sequences. Koichi Miyake demonstrated application of viral vector mediated siRNA expression for creating disease models and treatment. John M. Robinson showed placental proteomics and short hairpin RNA (shRNA) technology for functional analysis of proteins identified in human placenta. Yoel Sadovsky gave a lecture on integrated analysis of placental macroRNA and microRNA (miRNA) expression. Masahiro Seike revealed miRNA signatures in lung cancer. Finally, Yasunori Aizawa presented "long" noncoding RNAs (ncRNAs) from intergenic regions of the human genome. The studies discussed here provide new insights into basic and clinical medicine.

Effective and Target-specific Gene Silencing Regulated by SiRNA Sequences

Small regulatory RNAs, including siRNAs and miRNAs, are crucial regulators of gene expression at the posttranscriptional level[1,2]. RNA silencing encompasses a group of mechanistically related pathways that produce small ncRNA molecules, which modulate protein expression via mRNA degradation or translational repression. In RNA interference (RNAi), long double-stranded RNAs are processed by the cytoplasmic RNase III enzyme Dicer into 21- to 23-nucleotide siRNAs. The siRNA guide strand that is incorporated into the RNA-induced silencing complex (RISC) recognizes closely or completely matched target sequences, and a core component of RISC, the Argonaute (Ago) protein, mediates the sequence-specific cleavage of target mRNAs[3].

The siRNA-based RNAi has been used as a research tool to control the expression of specific genes in numerous experimental organisms and has potential as a therapeutic strategy for several human diseases. However, it has not been usable for the large-scale gene silencing essential for mammalian functional genomics, because only a limited fraction of siRNAs appear capable of producing highly effective RNAi in mammalian cells. Thus Ui-Tei and colleagues investigated the relationship between siRNA sequence and RNAi effect in mammalian cells[4]. They presented principles that govern siRNA sequence preference, by which highly effective siRNAs can be readily designed. The rules indicate that siRNAs that simultaneously satisfy all 4 of the following sequence conditions are capable of inducing highly effective gene silencing in mammalian cells: (i) A/U at the 5′ end of the guide strand; (ii) G/C at the 5′ end of the passenger strand; (iii) at least 5 A/U residues in the 5′ terminal one-third of the guide strand; and (iv) the absence of any GC stretch of more than 9 nucleotides in length. For 97.7% of mRNA sequences registered in the National Center for Biotechnology Information Reference Sequence (RefSeq) database, at least 1 effective siRNA sequence has been designed[5]. Essentially the same rules for siRNA sequence preference were found applicable to DNA-based RNAi in mammalian cells. However, a growing body of evidence from large-scale knockdown experiments suggests that siRNA could generate off-target effects through a mechanism similar to that of target silencing by miRNAs[6,7]. The 3′ untranslated regions (3′ UTRs) of off-target transcripts or miRNA targets are complementary to the seed region of the guide strand, nucleotide positions 2 to 8 measured from the 5′ end. Within cells, the siRNA guide strand binds to Ago to form RISC. In RISC, the seed nucleotides are presumed to be present on the surface of Ago in a quasihelical form to serve as the entry or nucleation site for mRNA[8]. However, little was known about the molecular basis that determines the efficiency of seed-dependent gene silencing by siRNA and miRNA. To clarify this point, Ui-Tei and colleagues examined the relationship between the stability of the seed-target duplex and the capability of siRNA to induce off-target effects[9]. They found that the capability of siRNA to induce off-target effects correlated strongly with the calculated melting temperature or standard free energy change for formation of the protein-free seed-target duplex. The thermodynamic stabilities of the duplexes were calculated from the seed sequences, indicating that the seed sequence is the major factor in determining

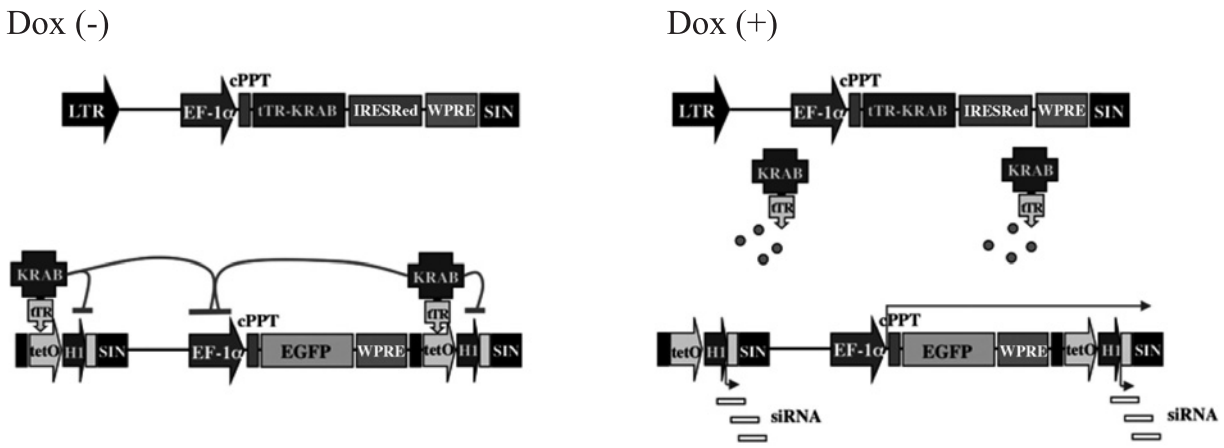

Fig. 1 Drug-inducible expression system of siRNA using lentiviral vectors.
Abbreviations: *Kruppel-associated box*, KRAB; *Self-inactivating LTR*, SIN.

the degree of off-target gene silencing. Furthermore, unlike RNAi, off-target effects were completely abolished by introduction of a G : U wobble pair into the seed-target duplex, indicating that Watson-Crick pairing in the seed-target duplex is also essential for off-target gene silencing. In this case, off-target effects are likely avoided because of the structural perturbation of the seed-target duplex on the surface of Ago protein but not the duplex stability.

Unlike the off-target gene silencing, intended gene silencing by RNAi was significantly tolerant to the presence of a G : U pair in the seed region. Ui-Tei and colleagues, therefore, consider that for RNAi, which requires a near-perfect match between target mRNA and the siRNA guide strand, the first recognition of mRNA by the seed region in the guide strand and the subsequent target recognition by the nonseed region of the guide strand are highly cooperative, and, therefore, the second proof-reading process can easily surmount the G : U pairing hurdle in the initial mRNA nucleation process.

Ui-Tei and colleagues presented that effective and target-specific gene silencing is closely related to the siRNA sequence, and it was found that >94% of human mRNAs in the RefSeq database possess at least 1 target sequence capable of producing: 1) functional siRNAs that satisfy these guidelines, 2) siRNAs with reduced seed-dependent off-target effects by considering the thermodynamic stability of the seed-target duplex, and 3) siRNAs that do not hit any nontargeted genes with near-perfect matches[10]. Thus, their procedure to select siRNA should be applied to diverse mammalian RNAi experiments including functional genomics.

Application of Viral Vector Mediated SiRNA Expression for Creating Disease Models and Treatment

SiRNAs have been shown to inhibit endogenous genes in a sequence-specific manner in mammalian cells[11,12]. Posttranscriptional gene silencing by siRNAs has quickly emerged as a powerful tool for genetic analysis in mammalian cells and has the potential to produce novel therapeutics. Miyake and colleagues have developed a novel regulatable expression system of siRNA using lentiviral vectors that can stably transduce both dividing and nondividing target cells[13]. This system can achieve the highly efficient, drug-inducible knockdown of cellular genes (**Fig. 1**). Using this lentiviral-mediated drug-inducible system, Miyake *et al.*[14] and Flygare *et al.*[15] have established cell-line models for ribosomal protein S19 (RPS19)-deficient Diamond-Blackfan anemia (DBA), as no models exist for RPS19-deficient DBA, and the molecular pathogenesis is unknown. To establish an in vitro inducible model for DBA, human erythroid leukemic cell lines TF-1 and UT-7 cells were cotransduced with a lentiviral vector expressing the green fluorescent protein (GFP) gene and an siRNA against RPS19 controlled by a tet operator regulatory element and another transactivator

vector containing the red fluorescent protein (RFP) gene and a cDNA encoding a tetracycline-controllable transcriptional repressor. Following transduction, the RFP-positive and GFP-negative cell population was isolated with flow cytometry. Upon incubation with doxycycline, more than 98% of cells expressed GFP and the siRNA. Significant suppression of erythroid differentiation, cell growth, and colony formation was observed in cells treated with the siRNA against RPS19 but not in cells treated with a control vector. These novel cell lines represent models for RPS19-deficient DBA and can be used to identify the molecular mechanisms in RPS19-deficient DBA[16]. Thus, this novel regulatable expression system of siRNA using lentiviral vector is useful for the knockdown of cellular genes.

Another potential viral vector to express siRNA is adeno-associated viral (AAV) vector[17,18]. Systemic gene delivery by AAV vectors has been successfully used for long-term expression of transgenes to treat genetic diseases and cancer. Recently, a number of novel AAV serotypes have been isolated from nonhuman primates. To analyze the utility of AAV vectors *in vivo*, Miyake and colleagues examined the expression patterns and time course of expressions for different serotypes of AAV[19] after the injection of the vectors into mice. They injected AAV vectors (types 1, 2, 4, 5, 6, 7, 8, 9, and 10: 1×10^{11} vector genome each) encoding luciferase gene into the tail vain of DDY mice and performed real-time monitoring of transgenes with a bioluminescent *in vivo* imaging system. The highest luciferase expression level in the whole body was observed in mice 1 week after receiving injections of AAV-8 (8> 9>7>10>6>5>1>4>2). The vectors AAV-1, -6, -7, -8, -9, and -10 induced rapid expression and reach a plateau at 4 weeks, whereas vectors AAV-2, -4, and -5 were slower to induce expression of the reporter gene. Expression was prominent in the liver, muscle, and heart in mice receiving injections of vectors AAV-1, -2, -5, -6, -7, -8, -9, -10, and in the lung of mice receiving injections of AAV-4. Miyake and colleagues also analyzed the transduction efficiency of several AAV vectors expressing luciferase and/or GFP gene by direct injection into brain, eye, and muscle[20]. The highest expression level in the brain, eye, and muscle was observed in AAV-9, AAV-8, and AAV-8 injected mice respectively. Because the AAV vector is nonpathogenic and can transduce with high efficiency in a wide variety of organs *in vivo*, the appropriate AAV vector serotype is a powerful tool for the delivery *in vivo* of siRNA and/or miRNA.

Placental Proteomics: a Gateway to Functional Analysis

Patterns of protein expression in tissues, cells, or subcellular components are characteristic of specific physiological, developmental, or pathological conditions. Therefore, the understanding of protein expression profiles of a sample under specific conditions is highly desirable. Proteomics refers to the identification and characterization of protein components in a biological sample. Ideally, the proteins would be quantified, and their posttranslational state determined. While the human genome has about 25,000 genes, the repertoire of protein expression in humans may be closer to 1 million[21]. This dramatic amplification of the protein repertoire is due to alternative splicing of genes and posttranslational modifications to which proteins are subject.

A detailed analysis of protein expression in healthy placentas is essential because the protein repertoire of the placenta is not known. These baseline data are essential for studies comparing normal and diseased placentas. However, global proteomics analysis of a complex structure, such as the placenta, is not feasible currently[22]. The best available strategy to deal with this problem is to simplify the placenta by fractionation; that is, to analyze subproteomes. Robinson and colleagues have focused on the maternal-placental interface subproteome, the apical plasma membrane of the syncytiotrophoblast (STB) of the human placenta. Specifically, they have focused on the membrane proteins in that subproteome. Defining this proteome is desirable considering the importance of this membrane to placenta function.

Enrichment of the apical plasma membrane of the STB to the greatest extent possible prior to the proteomics analysis is important. The need for

enrichment is due largely to the dynamic range of protein expression in biological samples. In serum, the dynamic range can vary over 12 orders of magnitude; in cells, the range can be over 8 orders of magnitude. This presents a practical problem for identifying low-abundance proteins[23]. Robinson and colleagues have used a procedure that is a modification of one used for the proteomic analysis of the luminal plasma membrane of rat lung endothelium[24]. In this procedure, the apical plasma membrane of the STB is coated with cationic colloidal silica, which alters the buoyant density of this membrane relative to the other membranes in the placenta. Following tissue homogenization, the apical membrane (actually microvilli) is isolated by centrifugation. A detailed account of this method was recently published[25]. Using this procedure, Robinson and colleagues were able to enrich for placental alkaline phosphatase, the classic marker for the apical plasma membrane of the STB, by 200 to 400 times. They then subjected the isolated microvilli to a series of extraction steps designed to dissociate noncovalent protein-protein interactions to further enrich for membrane proteins. Incubating the sample in environments of low salt, high salt, high pH, and urea resulted in a further 5-fold enrichment. There was 1,000- to 2,000-fold enrichment in the final sample. No other tissue plasma membrane has been enriched to this degree. This material was used for proteomics analysis by tandem mass spectrometry.

More than 500 proteins were identified in the enriched apical plasma membrane of the STB. These proteins were characterized in a number of ways. Robinson and colleagues were particularly interested in those identified proteins not previously known to be in the placenta. They have validated the presence of a number of these proteins using immunoblot analysis. It is important to confirm the mass spectrometry data using independent methods, such as immunoblotting.

Robinson and colleagues identified 68 proteins heretofore unknown in the human placenta. Their experimental attention has focused on 2 of these, dysferlin and myoferlin. Before their study, dysferlin and myoferlin were known to be only in skeletal muscle. Mutations of dysferlin lead to limb girdle muscular dystrophy and Myoshi's myopathy[26,27]. Moreover, experimental evidence indicates that dysferlin can serve to repair damaged plasma membranes in skeletal muscle[28,29]. The functions of dysferlin and myoferlin in the STB are not known. Robinson and colleagues have hypothesized that dysferlin and, perhaps, myoferlin serve a similar role in STB repair. This hypothesis is not easily addressed experimentally in the human placenta. They have turned to primary cytotrophoblasts isolated from term placenta and cell lines, such as BeWo, to address this question. They have found that in cytotrophoblasts and BeWo cells dysferlin is expressed after cell-cell fusion but not in mononuclear cells. This is similar to the placenta, where the STB expresses dysferlin but the cytotrophoblast does not[30,31]. Using shRNA technology, Robinson and colleagues have generated lines of BeWo cells lacking dysferlin, lacking myoferlin, or lacking both proteins. Robinson and colleagues are now studying these cell lines to determine the roles of dysferlin and myoferlin in trophoblast biology.

Integrated Analysis of Placental MacroRNA and MiRNA Expression

Intact function of the placenta is essential for the growth and development of the embryo. Within the placenta, the trophoblast layer governs gas exchange, nutrition, waste removal, endocrine function, and immunological support for the developing fetus. Sadovsky's research goal is to understand the process of human trophoblast differentiation during the latter half of pregnancy and the response of human trophoblast to injury. The consequences of abnormal trophoblast differentiation and placental dysfunction are far reaching, typically leading to fetal growth restriction or even fetal death, and frequently a need for medically indicated preterm delivery designed to prevent further damage[32]. Newborns surviving these insults are at risk for lifelong complications, including neuro-developmental dysfunction and a greater incidence of the adult metabolic syndrome

(including type 2 diabetes, hyperlipidemia, obesity, and related illnesses)[33]. Whereas the association of these conditions with placental dysfunction has been substantiated, the mechanisms underlying trophoblast differentiation and adaptation to injury are poorly understood. Furthermore, there is no effective treatment for this condition. The only intervention to prevent further fetal damage is delivery, which commonly leads to iatrogenic prematurity.

Diverse pathways coalesce to establish implantation and early placental development. Later in pregnancy, a different set of signals determines the differentiation and function of the established placenta. Exposure of the placenta to insults, such as hypoperfusion and cellular hypoxia, adversely affects trophoblast function and leads to placental injury. Hypoxia regulates trophoblast gene expression and adversely affects cell survival, differentiation, and metabolic function, which contribute to the pathophysiology of placental dysfunction and fetal growth restriction[34,35].

The noncoding, 20- to 24-nucleotide-long miRNAs are abundant in all metazoan eukaryotes, where they act posttranscriptionally to silence gene expression[36,37]. These small RNAs are processed from longer transcripts carrying a characteristic hairpin secondary structure. Currently, the number of validated mature human miRNAs approaches 1 thousand, with individual miRNAs capable of repressing multiple genes. Not surprisingly, miRNAs have emerged as important regulators of virtually every biological process associated with tissue development, differentiation, cellular proliferation, cell-type specific function, and homeostasis. Consequently, dysregulation of miRNAs has been implicated in the dysfunction of regulatory networks. Indeed, several pathologies have been linked to altered expression of miRNA[38,39].

Hypoxia was recently shown to impact miRNA expression in diverse cellular contexts. Sadovsky and colleagues have previously shown that the miRNA biosynthetic pathway is functional in human trophoblasts exposed to hypoxic stress and that the expression of key miRNA machinery proteins is not altered by hypoxia[40]. They now seek to understand the impact of hypoxia on trophoblast miRNAs and to assess their molecular targets. Using miRNA microarrays they defined a set of miRNAs (such as *miR-205*) that exhibits altered expression in hypoxic human trophoblasts. To define the targets of these miRNA they combined high-throughput profiles of miRNAs and mRNAs from hypoxic trophoblasts with *in silico* prediction of miRNA targets. Using this computational approach they identified candidate trophoblast target mRNAs to their hypoxia-induced mRNAs. They further confirmed miRNA targets using miRNA reporter technology and mutagenesis of specific miRNA binding elements[41]. Sadovsky and colleagues also assayed the level of these miRNAs in the plasma of women with pregnancy complicated by fetal growth restriction.

MiRNA Signatures in Lung Cancer

Lung cancer is the leading cause of cancer death in Japan and worldwide. The identification of sensitive and specific biomarkers for prognosis and drug sensitivity may have a significant impact on lung cancer treatment strategies.

MiRNAs are small noncoding RNA molecules comprising 18 to 25 nucleotides which are frequently located at previously reported regions for genetic alterations in cancers, suggesting that miRNAs are a new class of genes involved in human tumorigenesis. Recently, miRNAs have also been demonstrated to be diagnostic and prognostic biomarkers, including a role in monitoring the response to therapy in lung cancer[42,43]. For example, high *miR-155* expression and low *let7a* expression, as independent risk factors, have a negative prognostic impact in patients with lung adenocarcinoma[42]. Inhibition of the *miR-21* by enhanced epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI)-induced apoptosis in lung cancer cells, which showed intermediate sensitivity to the EGFR-TKI[43]. In miRNA-drug correlation analyses, Seike and colleagues have identified unique miRNA expression patterns that correlate with the drug sensitivity to each of commonly used 10 anticancer agents. These findings suggest that miRNAs may serve as novel diagnostic markers and therapeutic targets in lung cancer.

NcRNAs from Intergenic Regions of the Human Genome

Recent mammalian transcriptome analyses have uncovered tens of thousands of novel transcripts[44–46]. These transcripts differ in size, spliced mode, polyadenylation, status and intracellular location. Because their functional roles are currently unclear, they are designated as transcripts of unknown function (TUFs)[47,48]. In the postgenomic era, the functional significance of TUFs has received considerable attention.

A common feature of TUFs is low coding potential. In sharp contrast with mRNAs, which generally contain a single large open reading frame (ORF), TUFs have many start and stop codons scattered throughout the entire nucleotide sequences. Thus, TUFs always have several small ORFs and yet rarely resemble any protein families. Owing to these features, it is widely believed that most TUFs are "long" ncRNAs. For more than a decade, long and polyadenylated ncRNA genes, such as *XIST* (X (inactive)-specific transcript), have been known to be functionally essential to gene regulation on chromosomal and global scales[49]. Recently, the number of functional ncRNAs has been expanded using different experimental approaches[50]. This growing list of functional long ncRNAs does not, however, exclude the possibility that any TUFs encode small proteins. Indeed, it was found that novel and small proteins were encoded in transcripts that were originally believed to be ncRNAs in invertebrates. Therefore, one can expect that mammalian TUFs include unprecedented types of RNA and/or protein genes.

To gain an overview of biologically active mammalian TUFs, Aizawa and colleagues screened for candidates of functional TUFs and then investigated sequences of the identified TUFs. This study focused on human TUFs that are involved in multilineage differentiation of human mesenchymal stem cells (hMSCs). In general, stem cells are much more transcriptionally active than are differentiated cells[51], and induction of differentiation triggers global and stepwise activation and/or inactivation of many genes and signaling pathways[52,53]. Thus, Aizawa and colleagues expected that some TUFs were composed of signaling networks for stem cell differentiation and dynamically changed transcription activities while differentiation proceeds. On the basis of this speculation, they designed a DNA microarray for approximately 5,500 human TUFs and monitored the transcription levels for the first 7 days of hMSC adipogenesis and osteogenesis[54]. Consequently, Aizawa and colleagues obtained 6 strong candidates of functional TUFs, which exhibited marked and lineage-specific changes in abundance.

Subsequent sequence analysis of the resultant TUFs allowed us to unexpectedly find 1 of the 6 TUFs, named adipogenesis downregulating gene 3 (AGD3). Among the 6 TUFs, AGD3 is the only one that has been reported in the literature and was found to be highly expressed in colon cancer as a ncRNA[55]. However, by examining orthologous transcripts obtained from the human AGD3 syntenic regions in the mouse, chicken, and zebrafish genomes, Aizawa and colleagues demonstrated that all these AGD3 orthologs contain small sequence-homologous ORFs. They then prepared polyclonal antibody against AGD3-derived peptides and confirmed with Western blotting that the protein was indeed produced in hMSCs[54]. To our knowledge, AGD3 is the first mammalian TUF that was experimentally shown to be translated. The subsequent pathway analysis using protein kinase A (PKA) inhibitor compounds (3-isobutyl-1-methylxanthine and forskolin) showed that AGD3 expression is suppressed during hMSC adipogenesis by the PKA pathway. With the observed up-regulation in colon tumors, this finding may suggest that AGD3 gene is invovled in cell proliferation.

Serendipitous finding of AGD3 brought up a fascinating hypothesis: TUFs could be a valuable reservoir of new protein genes. As mentioned above, ORFs on TUFs are all small (<100 amino acids), and the amino acid sequences are rarely categorized into any known protein families. Therefore, TUF-originated protein genes would be able to create new protein families. Indeed, on the basis of the sequence features of the AGD3 transcription unit,

Aizawa and colleagues carried out a pilot screening of human TUFs for new protein genes and discovered several protein gene candidates, which all have orthologs in mammals or vertebrates but no sequence similarities with any known protein families. Future studies may demonstrate as-yet-unidentified molecular mechanisms that these new proteins govern in cells.

Future Directions

The technological advances and applications of functional RNA molecules for medicine discussed in the symposium provide important insights into molecular mechanisms affecting human health and disease and should eventually lead to the discovery of diagnostic biomarkers and the development of novel gene therapies.

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