
DYSTROGEN
THERAPEUTICS
Regenerative Medicine Life Sciences Company Focusing
on Rare Diseases
RNAi Platform

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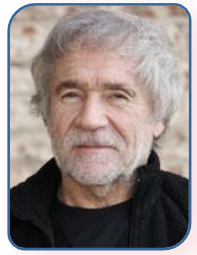

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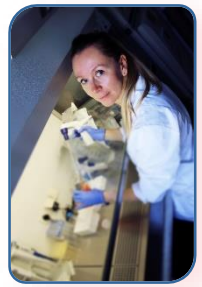

Prof. Natalia
Rozwadowska, PhD
Chief Scientific Officer

Development Strategy Routes

two independent technology platforms

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PATENT US20180221416A1

Cellular Therapy Platform

Rare diseases:

- Duchenne Muscular Dystrophy
- Anti-aging
- Sickle Cell Disease
- Collagenopathies
- GvHD

PATENT US9970004B2

RNAi Platform

Neurodegenerative diseases:

- Huntington's Disease
- Machado-Joseph disease
- DRPLA
- SCA7

Dystrogen Therapeutics Pipeline

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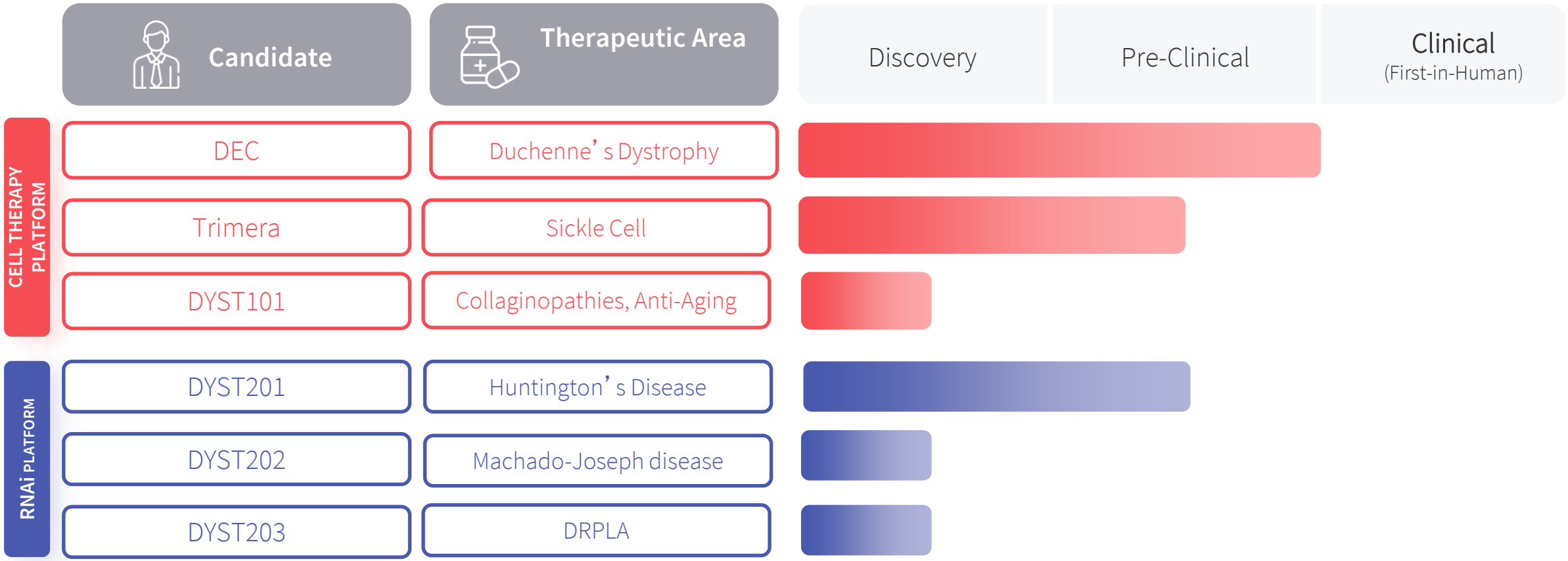

RNAi Platform
Therapy for Huntington's Disease
and other Polyglutamine (polyQ) Diseases

RNA Platform for Huntington’s Disease Orphan Diseases

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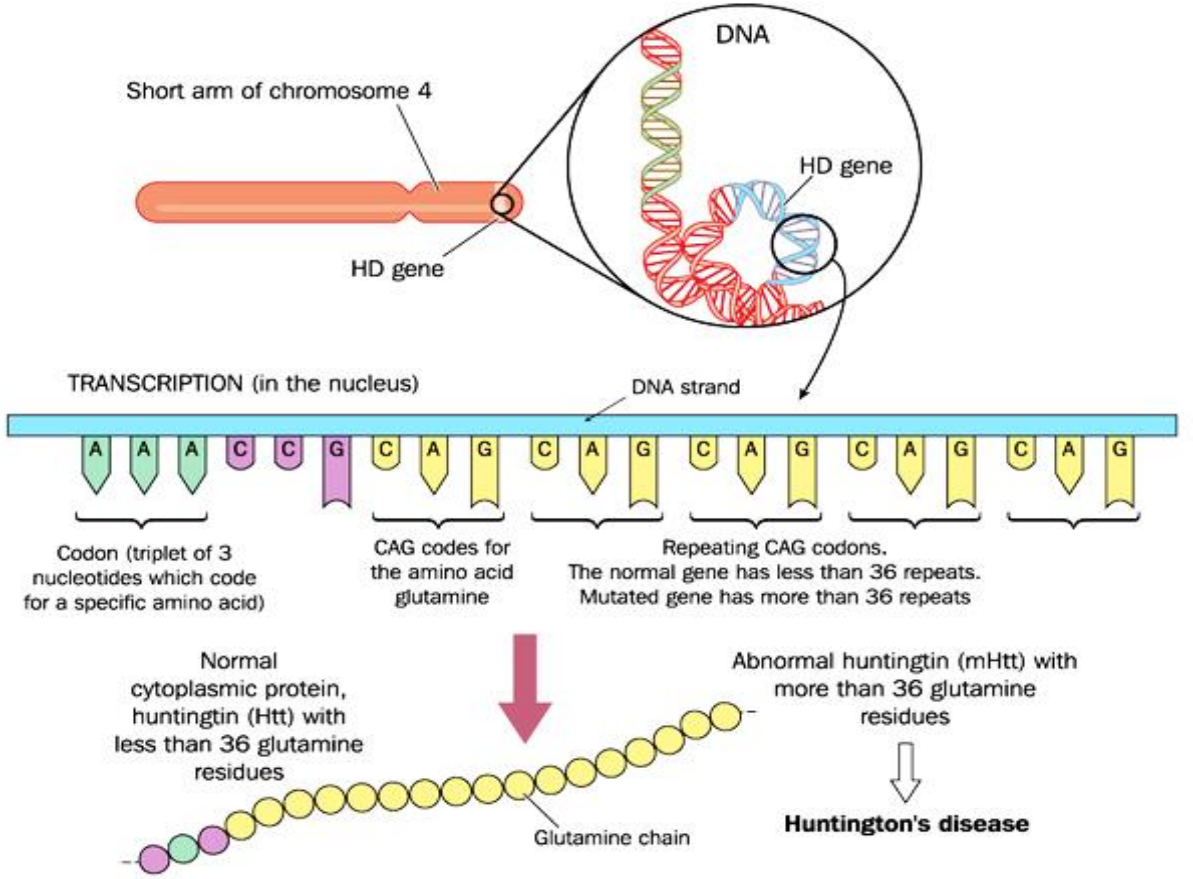
- Rare disease, caused by an inherited defect in a single gene
- Huntington's disease (HD) is genetic disorder that results in death of brain cells
- Symptoms usually begin between 30 and 50 years of age
- Prognosis: 15–20 years from diagnosis
- Frequency: 4–15 in 100,000 in Europe
- Universal therapy with selective silencing of gene expression („Allele-selectivity”), and long-term silencing effect



Dystrogen's RNA Interference Technology as Potential Therapy for Polyglutamine Diseases

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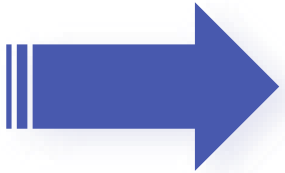
- Huntington`s Disease (HD)
- Spinal & Bulbar Muscular Atrophy (SBMA, Kennedy disease)
- Dentatorubral Pallidoluysian Atrophy (DRPLA)
- Spinocerebellar Ataxia Type 1 (SCA1)
- Spinocerebellar Ataxia Type 2 (SCA2)
- Spinocerebellar Ataxia Type 3 (SCA3, Machado-Joseph disease)
- Spinocerebellar Ataxia Type 6 (SCA6)
- Spinocerebellar Ataxia Type 7 (SCA7)
- Spinocerebellar Ataxia Type 17(SCA17)



Human diseases caused by CAG repeat expansions (polyglutamine diseases)

Product Composition

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UNIQUE
NUCLEIC ACID MOLECULE

+

EXPRESSION VECTOR

VIRAL- based
VECTOR

Mode of Action (intrathecal route of administration)

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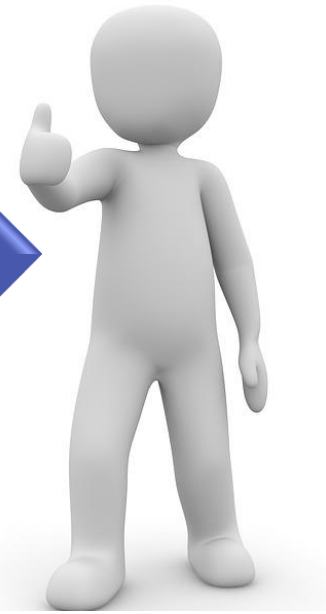
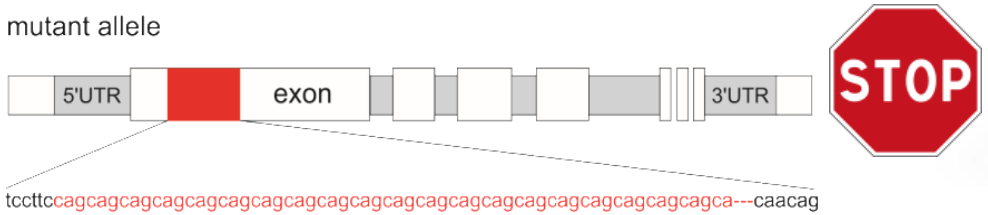
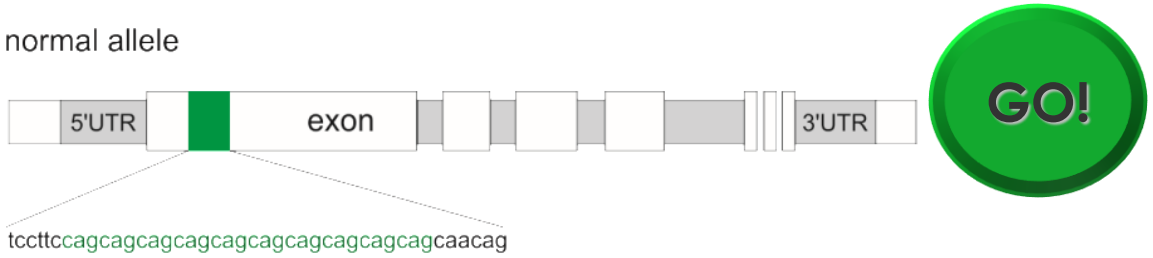
Viral-based RNAi → Intrathecal route of administration → Selective silencing of the mutant gene → Inhibition of neurodegeneration



Long acting therapy!





Competitive Advantagen of RNA Therapy

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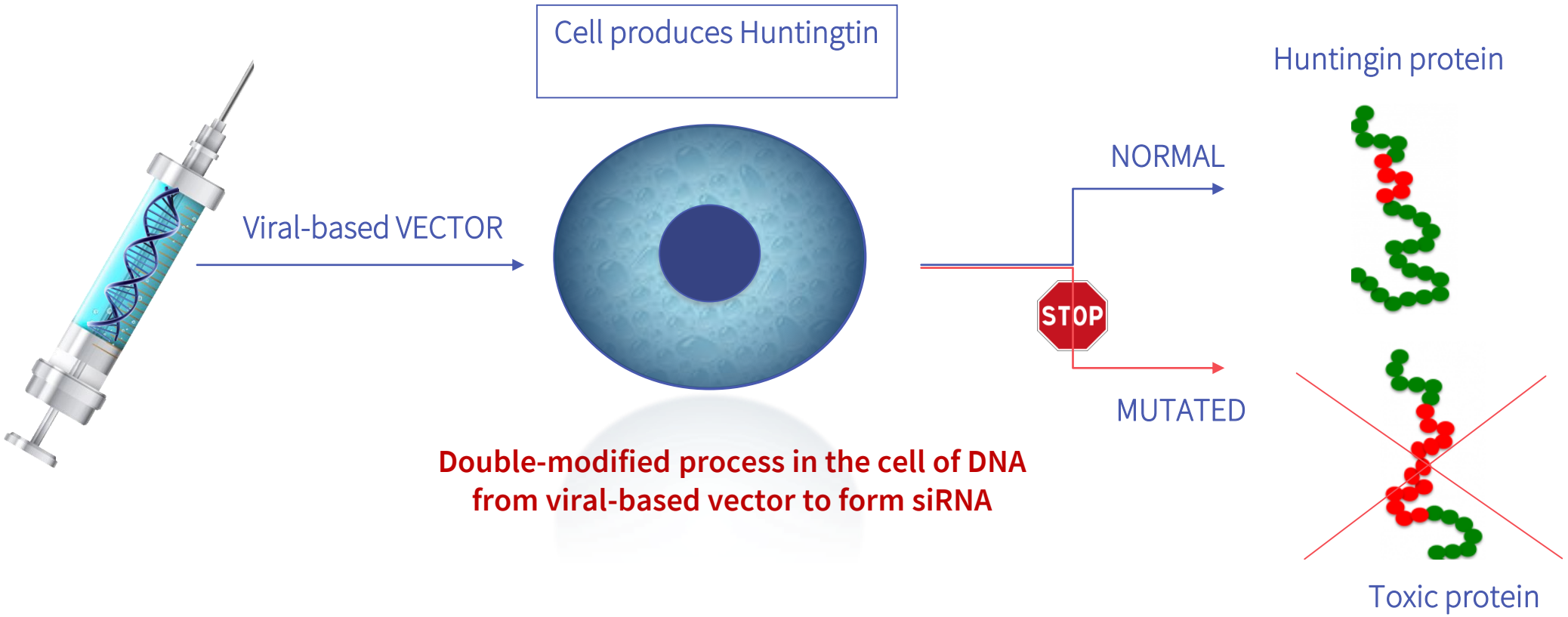
GENE THERAPIES	CAG sh-miRNA	Exon-targeting	SNP- targeting
Allele-selectivity	Acting only on the mutant gene	NO	Acting only on the mutant gene
Technology	RNAi	RNAi	Antisense
Target	Mutant CAG repeats	Specific sequence	SNP1 and SNP2
Targets HD patients	100% Universal Therapy	100%	Treatment for up to 70% of HD patients
Potential universal therapy for other diseases with CAG expansion	Yes	No	No
Mode of silencing	Long-term	Long-term	Short-term

Mechanism of Action: Selective Silencing

DYSTROGEN THERAPEUTICS

USES NATURAL CELLULAR MECHANISMS TO SILENCE EXPRESSION OF MUTANT HUNTINGTIN



Double-modified process in the cell of DNA from viral-based vector to form siRNA

Results of Selectively Silencing Expression of Mutant Genes on Protein Expression Levels

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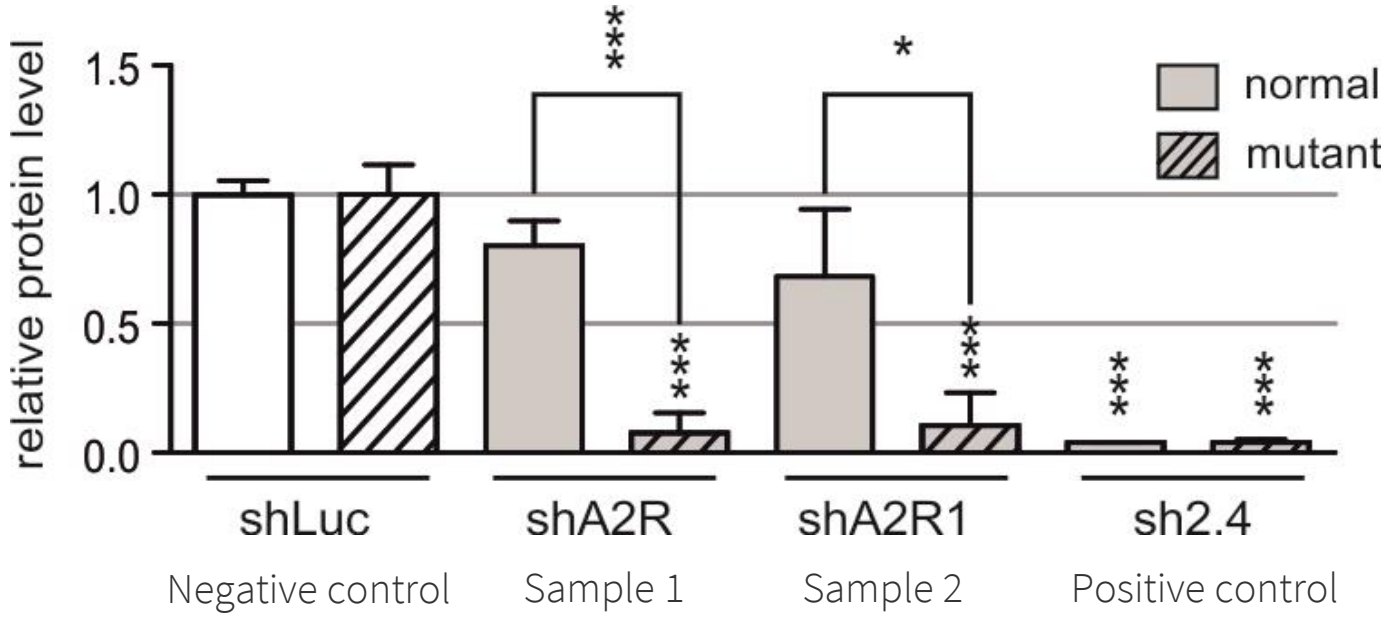

Western blot analysis demonstrated that shRNAs Sample 1 and Sample 2 efficiently reduced mutant huntingtin protein expression of about 90% of the control level and left the normal huntingtin gene intact.

Safe Tools for *in-vivo* Applications

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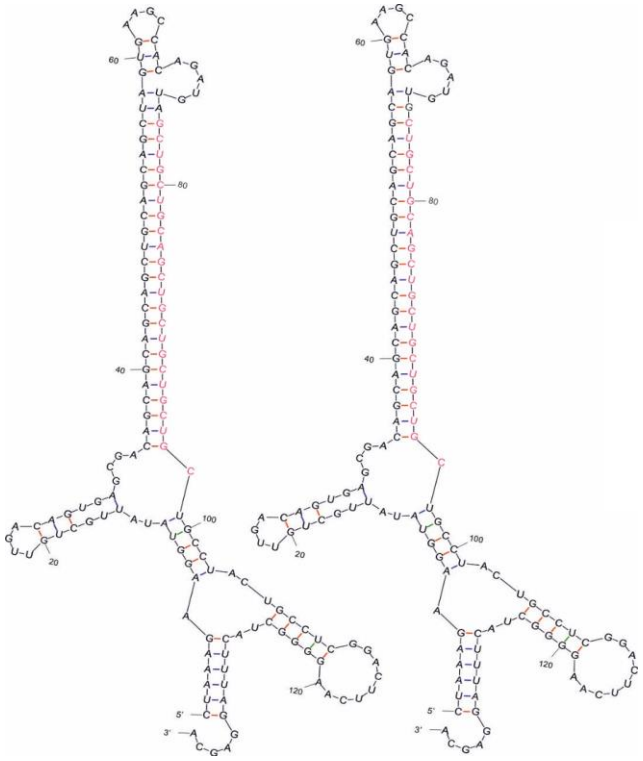
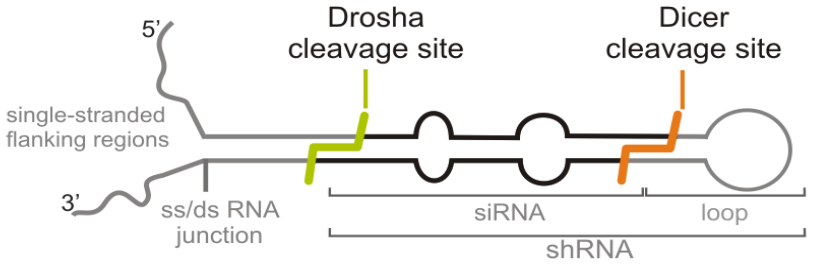
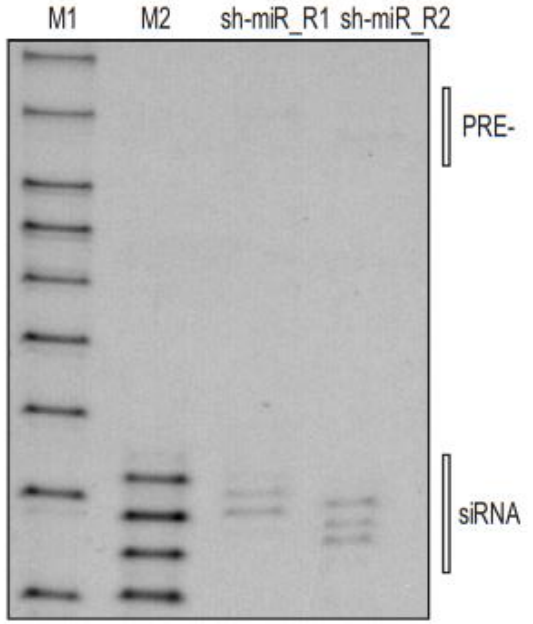
Interfering RNA molecules directed against mutant CAG pathways are cut by Dicer RNAse to the pool of short heterogeneous siRNA molecules.







Summary
Competitive Advantages of RNAi Therapy

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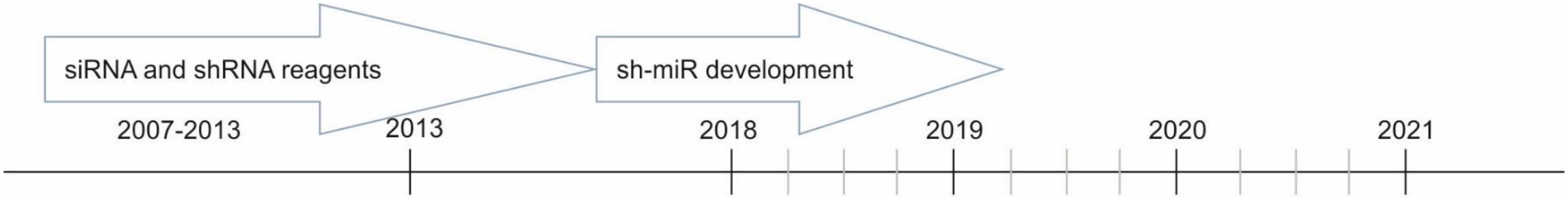
Our RNAi therapy provides:

- Selective inhibition of mutant allele expression making it a promising therapeutic option
- A new class of CAG repeat-targeting silencing reagents forming A:A or G:A mismatches between siRNA and a target transcript (translation inhibition mechanism)
- Preferential silencing of mutant allele of Huntingtin gene.
- Long-term silencing effect: active and selective therapy when expressed from short hairpin RNA and miRNA vectors to achieve more durable silencing effects; Active reagents can be expressed in cells from genetic vectors
- Active on 100% of Huntington's disease patients
- Potential universal therapy for other diseases with CAG expansion

Development Milestones

- Optimization of artificial miRNA (sh-miR) constructs
- Preclinical study in HD mouse model



Development of selective RNAi triggers for HD

- patent application (2013)
- publication:
„Self-duplexing CUG repeats selectively inhibit mutant huntingtin expression"
Fiszer A, Olejniczak M, Galka-Marciniak P, Mykowska A, Krzyzosiak WJ.
Nucleic Acids Res. 2013;41:10426-37

Confirmation of activity in other polyQ models

- SCA7, Fiszer A. et al., 2016
- SCA3, Fiszer A. et al., 2016
- DRPLA, unpublished data

Preclinical study, mouse model

Preclinical study, model 2
- delivery, dose
- distribution
- toxicity

Intellectual Property – RNAi Platform

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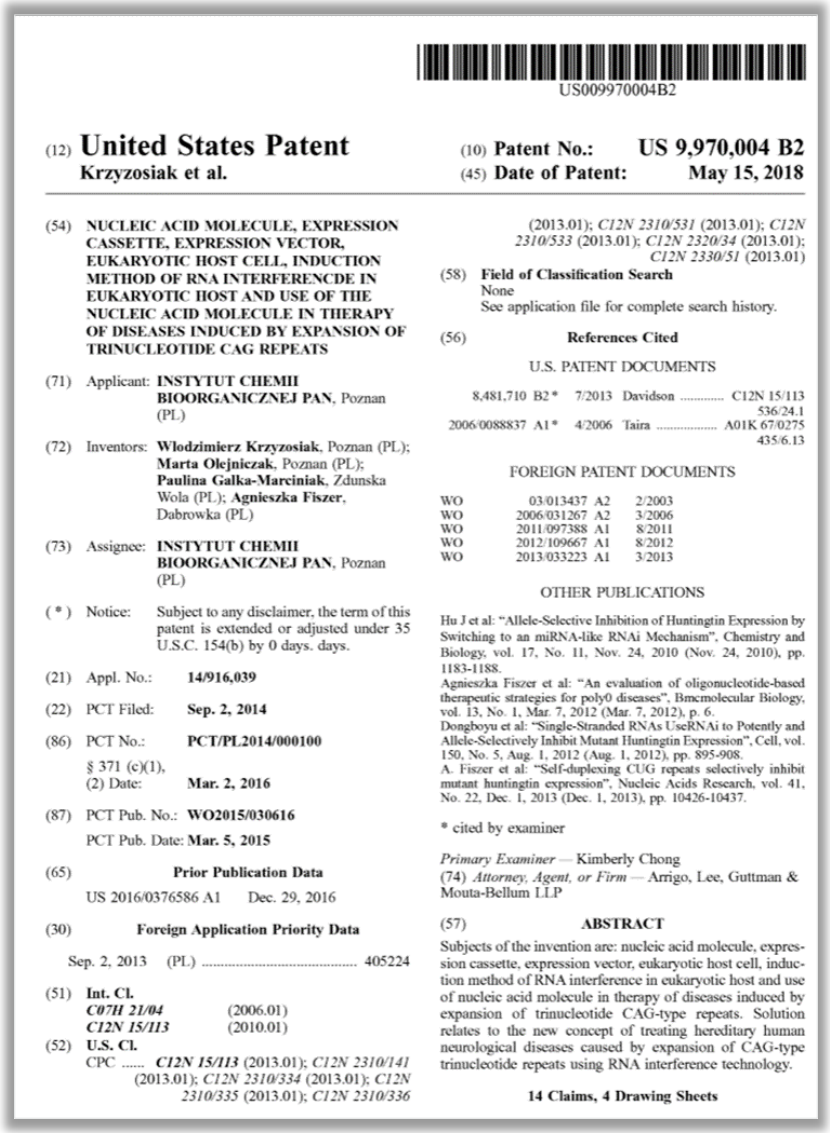
- US Patent granted US9970004B2
- EPO Patent Application no. EP 3041936
- Priority date: 2013-09-02
- PCT Application
- Title: Nucleic acid molecule, expression cassette, expression vector, eukaryotic host cell, induction method of RNA interference in eukaryotic host and use of nucleic acid molecule in therapy of diseases induced by expansion of trinucleotide CAG repeats
- Proprietary Technology

  Usage of RNAi technology vector reagents for selective silencing of mutant genes
- Exclusive Worldwide License From INST CHEMII BIOORG PAN Instytut Chemii Bioorganicznej Pan
- https://patents.google.com/patent/US9970004B2/un



US009970004B2

(12) **United States Patent**
Krzyzosiak et al.

(10) **Patent No.: US 9,970,004 B2**
(45) **Date of Patent: May 15, 2018**

(54) **NUCLEIC ACID MOLECULE, EXPRESSION CASSETTE, EXPRESSION VECTOR, EUKARYOTIC HOST CELL, INDUCTION METHOD OF RNA INTERFERENCDE IN EUKARYOTIC HOST AND USE OF THE NUCLEIC ACID MOLECULE IN THERAPY OF DISEASES INDUCED BY EXPANSION OF TRINUCLEOTIDE CAG REPEATS**

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(72) Inventors: **Wlodzimierz Krzyzosiak**, Poznan (PL); **Marta Olejniczak**, Poznan (PL); **Paulina Galka-Marciniak**, Zdunska Wola (PL); **Agnieszka Fiszer**, Dabrowka (PL)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days. days.

(21) Appl. No.: **14/916,039**

(22) PCT Filed: **Sep. 2, 2014**

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§ 371 (c)(1),
(2) Date: **Mar. 2, 2016**

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PCT Pub. Date: **Mar. 5, 2015**

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US 2016/0376586 A1 Dec. 29, 2016

(30) **Foreign Application Priority Data**
Sep. 2, 2013 (PL) 405224

(51) **Int. Cl.**
C07H 21/04 (2006.01)
C12N 15/113 (2010.01)

(52) **U.S. Cl.**
CPC ***C12N 15/113*** (2013.01); *C12N 2310/141* (2013.01); *C12N 2310/334* (2013.01); *C12N 2310/335* (2013.01); *C12N 2310/336* (2013.01); *C12N 2310/531* (2013.01); *C12N 2310/533* (2013.01); *C12N 2320/34* (2013.01); *C12N 2330/51* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Agnieszka Fiszer et al: "An evaluation of oligonucleotide-based therapeutic strategies for poly0 diseases", Bmcmolecular Biology, vol. 13, No. 1, Mar. 7, 2012 (Mar. 7, 2012), p. 6.
Dongboyu et al: "Single-Stranded RNAs UseRNAi to Potently and Allele-Selectively Inhibit Mutant Huntingtin Expression", Cell, vol. 150, No. 5, Aug. 1, 2012 (Aug. 1, 2012), pp. 895-908.
A. Fiszer et al: "Self-duplexing CUG repeats selectively inhibit mutant huntingtin expression", Nucleic Acids Research, vol. 41, No. 22, Dec. 1, 2013 (Dec. 1, 2013), pp. 10426-10437.

* cited by examiner

Primary Examiner — Kimberly Chong
(74) *Attorney, Agent, or Firm* — Arrigo, Lee, Guttman & Mouta-Bellum LLP

(57) **ABSTRACT**

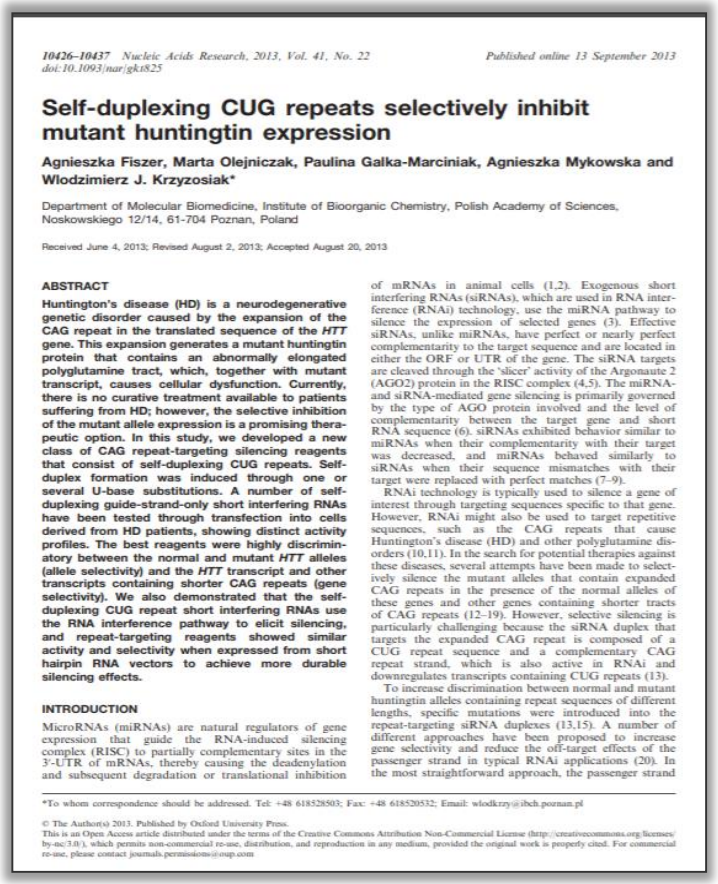
Subjects of the invention are: nucleic acid molecule, expression cassette, expression vector, eukaryotic host cell, induction method of RNA interference in eukaryotic host and use of nucleic acid molecule in therapy of diseases induced by expansion of trinucleotide CAG-type repeats. Solution relates to the new concept of treating hereditary human neurological diseases caused by expansion of CAG-type trinucleotide repeats using RNA interference technology.

14 Claims, 4 Drawing Sheets

Peer-Reviewed Publications
Professor Marta Olejniczak

DYSTROGEN THERAPEUTICS

https://www.ncbi.nlm.nih.gov/pubmed/24038471



10426–10437 Nucleic Acids Research, 2013, Vol. 41, No. 22 Published online 13 September 2013
doi:10.1093/nar/gkt825

Self-duplexing CUG repeats selectively inhibit mutant huntingtin expression

Agnieszka Fiszer, Marta Olejniczak, Paulina Galka-Marciniak, Agnieszka Mykowska and Wlodzimierz J. Krzyzosiak*

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ABSTRACT

Huntington's disease (HD) is a neurodegenerative genetic disorder caused by the expansion of the CAG repeat in the translated sequence of the *HTT* gene. This expansion generates a mutant huntingtin protein that contains an abnormally elongated polyglutamine tract, which, together with mutant transcript, causes cellular dysfunction. Currently, there is no curative treatment available to patients suffering from HD; however, the selective inhibition of the mutant allele expression is a promising therapeutic option. In this study, we developed a new class of CAG repeat-targeting silencing reagents that consist of self-duplexing CUG repeats. Self-duplex formation was induced through one or several U-base substitutions. A number of self-duplexing guide-strand-only short interfering RNAs have been tested through transfection into cells derived from HD patients, showing distinct activity profiles. The best reagents were highly discriminatory between the normal and mutant *HTT* alleles (allele selectivity) and the *HTT* transcript and other transcripts containing shorter CAG repeats (gene selectivity). We also demonstrated that the self-duplexing CUG repeat short interfering RNAs use the RNA interference pathway to elicit silencing, and repeat-targeting reagents showed similar activity and selectivity when expressed from short hairpin RNA vectors to achieve more durable silencing effects.

INTRODUCTION

MicroRNAs (miRNAs) are natural regulators of gene expression that guide the RNA-induced silencing complex (RISC) to partially complementary sites in the 3′-UTR of mRNAs, thereby causing the deadenylation and subsequent degradation or translational inhibition of mRNAs in animal cells (1,2). Exogenous short interfering RNAs (siRNAs), which are used in RNA interference (RNAi) technology, use the miRNA pathway to silence the expression of selected genes (3). Effective siRNAs, unlike miRNAs, have perfect or nearly perfect complementarity to the target sequence and are located in either the ORF or UTR of the gene. The siRNA targets are cleaved through the 'slicer' activity of the Argonaute 2 (AGO2) protein in the RISC complex (4,5). The miRNA- and siRNA-mediated gene silencing is primarily governed by the type of AGO protein involved and the level of complementarity between the target gene and short RNA sequence (6). siRNAs exhibited behavior similar to miRNAs when their complementarity with their target was decreased, and miRNAs behaved similarly to siRNAs when their sequence mismatches with their target were replaced with perfect matches (7–9).

RNAi technology is typically used to silence a gene of interest through targeting sequences specific to that gene. However, RNAi might also be used to target repetitive sequences, such as the CAG repeats that cause Huntington's disease (HD) and other polyglutamine disorders (10,11). In the search for potential therapies against these diseases, several attempts have been made to selectively silence the mutant alleles that contain expanded CAG repeats in the presence of the normal alleles of these genes and other genes containing shorter tracts of CAG repeats (12–19). However, selective silencing is particularly challenging because the siRNA duplex that targets the expanded CAG repeat is composed of a CUG repeat sequence and a complementary CAG repeat strand, which is also active in RNAi and downregulates transcripts containing CUG repeats (13).

To increase discrimination between normal and mutant huntingtin alleles containing repeat sequences of different lengths, specific mutations were introduced into the repeat-targeting siRNA duplexes (13,15). A number of different approaches have been proposed to increase gene selectivity and reduce the off-target effects of the passenger strand in typical RNAi applications (20). In the most straightforward approach, the passenger strand

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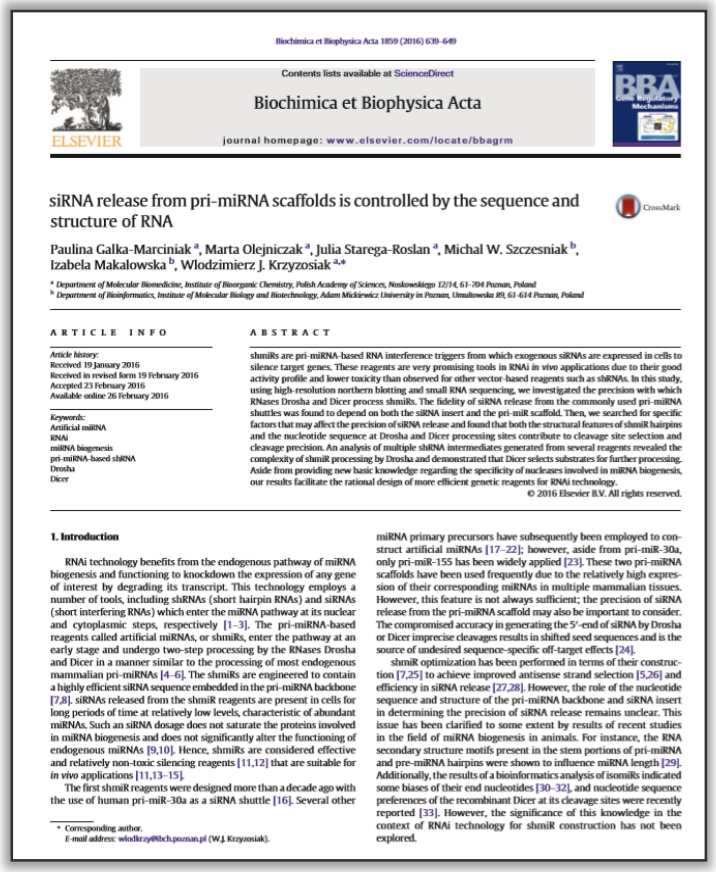

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journal homepage: www.elsevier.com/locate/bbagrm

siRNA release from pri-miRNA scaffolds is controlled by the sequence and structure of RNA

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miRNA biogenesis
pri-miRNA-based shRNA
Drosha
Dicer

ABSTRACT

shmiRs are pri-miRNA-based RNA interference triggers from which exogenous siRNAs are expressed in cells to silence target genes. These reagents are very promising tools in RNAi *in vivo* applications due to their good activity profile and lower toxicity than observed for other vector-based reagents such as shRNAs. In this study, using high-resolution northern blotting and small RNA sequencing, we investigated the precision with which RNases Drosha and Dicer process shmiRs. The fidelity of siRNA release from the commonly used pri-miRNA shuttles was found to depend on both the siRNA insert and the pri-miR scaffold. Then, we searched for specific factors that may affect the precision of siRNA release and found that both the structural features of shmiR hairpins and the nucleotide sequence at Drosha and Dicer processing sites contribute to cleavage site selection and cleavage precision. An analysis of multiple shRNA intermediates generated from several reagents revealed the complexity of shmiR processing by Drosha and demonstrated that Dicer selects substrates for further processing. Aside from providing new basic knowledge regarding the specificity of nucleases involved in miRNA biogenesis, our results facilitate the rational design of more efficient genetic reagents for RNAi technology.

1. Introduction

RNAi technology benefits from the endogenous pathway of miRNA biogenesis and functioning to knockdown the expression of any gene of interest by degrading its transcript. This technology employs a number of tools, including shRNAs (short hairpin RNAs) and siRNAs (short interfering RNAs) which enter the miRNA pathway at its nuclear and cytoplasmic steps, respectively [1–3]. The pri-miRNA-based reagents called artificial miRNAs, or shmiRs, enter the pathway at an early stage and undergo two-step processing by the RNases Drosha and Dicer in a manner similar to the processing of most endogenous mammalian pri-miRNAs [4–6]. The shmiRs are engineered to contain a highly efficient siRNA sequence embedded in the pri-miRNA backbone [7,8]. siRNAs released from the shmiR reagents are present in cells for long periods of time at relatively low levels, characteristic of abundant miRNAs. Such an siRNA dosage does not saturate the proteins involved in miRNA biogenesis and does not significantly alter the functioning of endogenous miRNAs [9,10]. Hence, shmiRs are considered effective and relatively non-toxic silencing reagents [11,12] that are suitable for *in vivo* applications [11,13–15].

The first shmiR reagents were designed more than a decade ago with the use of human pri-miR-30a as a siRNA shuttle [16]. Several other miRNA primary precursors have subsequently been employed to construct artificial miRNAs [17–22]; however, aside from pri-miR-30a, only pri-miR-155 has been widely applied [23]. These two pri-miRNA scaffolds have been used frequently due to the relatively high expression of their corresponding miRNAs in multiple mammalian tissues. However, this feature is not always sufficient; the precision of siRNA release from the pri-miRNA scaffold may also be important to consider. The compromised accuracy in generating the 5′-end of siRNA by Drosha or Dicer imprecise cleavages results in shifted seed sequences and is the source of undesired sequence-specific off-target effects [24].

shmiR optimization has been performed in terms of their construction [7,25] to achieve improved antisense strand selection [5,26] and efficiency in siRNA release [27,28]. However, the role of the nucleotide sequence and structure of the pri-miRNA backbone and siRNA insert in determining the precision of siRNA release remains unclear. This issue has been clarified to some extent by results of recent studies in the field of miRNA biogenesis in animals. For instance, the RNA secondary structure motifs present in the stem portions of pri-miRNA and pre-miRNA hairpins were shown to influence miRNA length [29]. Additionally, the results of a bioinformatics analysis of isomiRs indicated some biases of their end nucleotides [30–32], and nucleotide sequence preferences of the recombinant Dicer at its cleavage sites were recently reported [33]. However, the significance of this knowledge in the context of RNAi technology for shmiR construction has not been explored.

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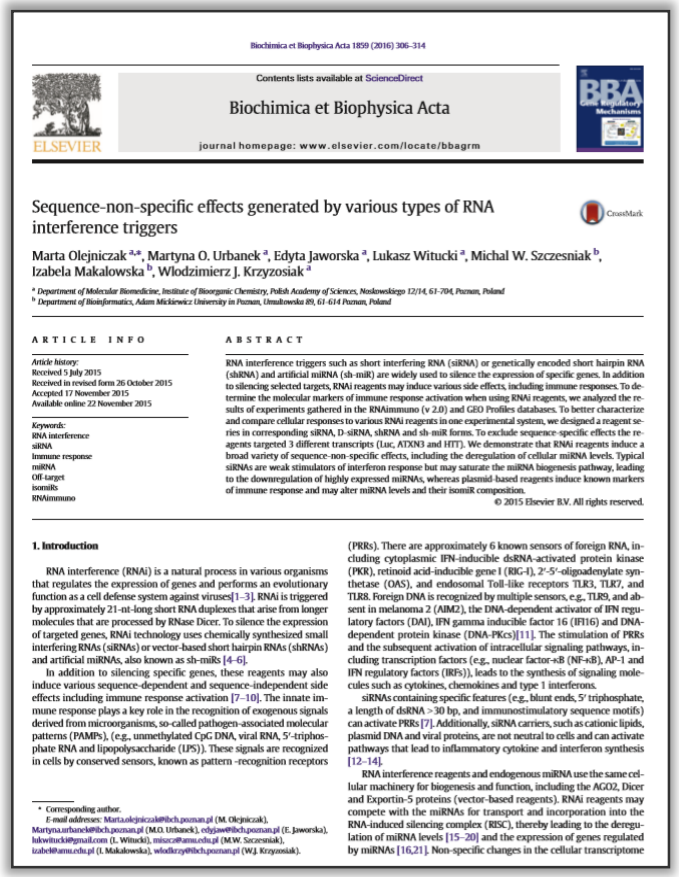

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Sequence-non-specific effects generated by various types of RNA interference triggers

Marta Olejniczak [a,*], Martyna O. Urbanek [a], Edyta Jaworska [a], Lukasz Witucki [a], Michal W. Szczesniak [b], Izabela Makalowska [b], Wlodzimierz J. Krzyzosiak [a]

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RNAimmuno

ABSTRACT

RNA interference triggers such as short interfering RNA (siRNA) or genetically encoded short hairpin RNA (shRNA) and artificial miRNA (sh-miR) are widely used to silence the expression of specific genes. In addition to silencing selected targets, RNAi reagents may induce various side effects, including immune responses. To determine the molecular markers of immune response activation when using RNAi reagents, we analyzed the results of experiments gathered in the RNAimmuno (v 2.0) and GEO Profiles databases. To better characterize and compare cellular responses to various RNAi reagents in one experimental system, we designed a reagent series in corresponding siRNA, D-siRNA, shRNA and sh-miR forms. To exclude sequence-specific effects the reagents targeted 3 different transcripts (Luc, ATXN3 and HTT). We demonstrate that RNAi reagents induce a broad variety of sequence-non-specific effects, including the deregulation of cellular miRNA levels. Typical siRNAs are weak stimulators of interferon response but may saturate the miRNA biogenesis pathway, leading to the downregulation of highly expressed miRNAs, whereas plasmid-based reagents induce known markers of immune response and may alter miRNA levels and their isomiR composition.

1. Introduction

RNA interference (RNAi) is a natural process in various organisms that regulates the expression of genes and performs an evolutionary function as a cell defense system against viruses[1–3]. RNAi is triggered by approximately 21-nt-long short RNA duplexes that arise from longer molecules that are processed by RNase Dicer. To silence the expression of targeted genes, RNAi technology uses chemically synthesized small interfering RNAs (siRNAs) or vector-based short hairpin RNAs (shRNAs) and artificial miRNAs, also known as sh-miRs [4–6].

In addition to silencing specific genes, these reagents may also induce various sequence-dependent and sequence-independent side effects including immune response activation [7–10]. The innate immune response plays a key role in the recognition of exogenous signals derived from microorganisms, so-called pathogen-associated molecular patterns (PAMPs), (e.g., unmethylated CpG DNA, viral RNA, 5′-triphosphate RNA and lipopolysaccharide (LPS)). These signals are recognized in cells by conserved sensors, known as pattern -recognition receptors (PRRs). There are approximately 6 known sensors of foreign RNA, including cytoplasmic IFN-inducible dsRNA-activated protein kinase (PKR), retinoid acid-inducible gene I (RIG-I), 2′-5′-oligoadenylate synthetase (OAS), and endosomal Toll-like receptors TLR3, TLR7, and TLR8. Foreign DNA is recognized by multiple sensors, e.g., TLR9, and absent in melanoma 2 (AIM2), the DNA-dependent activator of IFN regulatory factors (DAI), IFN gamma inducible factor 16 (IFI16) and DNA-dependent protein kinase (DNA-PKcs)[11]. The stimulation of PRRs and the subsequent activation of intracellular signaling pathways, including transcription factors (e.g., nuclear factor-κB (NF-κB), AP-1 and IFN regulatory factors (IRFs)), leads to the synthesis of signaling molecules such as cytokines, chemokines and type 1 interferons.

siRNAs containing specific features (e.g., blunt ends, 5′ triphosphate, a length of dsRNA > 30 bp, and immunostimulatory sequence motifs) can activate PRRs [7]. Additionally, siRNA carriers, such as cationic lipids, plasmid DNA and viral proteins, are not neutral to cells and can activate pathways that lead to inflammatory cytokine and interferon synthesis [12–14].

RNA interference reagents and endogenous miRNA use the same cellular machinery for biogenesis and function, including the AGO2, Dicer and Exportin-5 proteins (vector-based reagents). RNAi reagents may compete with the miRNAs for transport and incorporation into the RNA-induced silencing complex (RISC), thereby leading to the deregulation of miRNA levels [15–20] and the expression of genes regulated by miRNAs [16,21]. Non-specific changes in the cellular transcriptome

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