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Summary of dissertation

Účinky exprese dlouhé dvouvláknové RNA v savčích buňkách

The effects of long double-stranded RNA expression in mammalian cells

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Souhrn v českém jazyce

Dvouvláknová RNA je cizorodá molekula, která se objevuje v buňce buď jako produkt replikace viru nebo vzniká párováním komplementárních RNA, a to v rámci jedné molekuly nebo mezi dvěma molekulami. Komplementární RNA často pocházejí z repetitivních sekvencí. U savců může dvouvláknová RNA vstupovat do jedné ze tří drah: sekvenčně specifického umlčování RNA (RNA silencing), sekvenčně nezávislé interferonové (IFN) odpovědi nebo editace pomocí adenosin deamin——áz.

Hlavním zaměřením mého PhD projektu bylo detailně zanalyzovat efekty dvouvláknové RNA produkované v savčích buňkách, a to v rámci celého organismu. Za tímto účelem jsme vytvořili konstrukt exprimující dvouvláknovou RNA ve formě mRNA, obsahující dlouhou a perfektně párující vlásenkovitou strukturu.

Transgenní myši, vytvářející dvouvláknovou RNA ve všech buňkách, byly životaschopné a, na rozdíl od výsledků dosavadních studií, neměly aktivovanou IFN odpověď. V somatických buňkách byla dvouvláknová RNA jen málo štěpena na krátké interferující RNA (siRNA), nezpůsobovala transkripční umlčování v *trans* a docházelo k mírné deaminaci adenosinu bez zadržování editované RNA v jádře. Shodné výsledky jsme získali v savčích buňkách, které byly tranzientně transfekovány plazmidem exprimujícím dvouvláknovou RNA. Naproti tomu ve vajíčcích způsobovala dvouvláknová RNA silnou RNA interferenci. Naše výsledky jako první ukazují, ze exprimovaná dvouvláknová RNA, na rozdíl od mnoha jiných forem dvouvláknové RNA, je v savčích buňkách dobře tolerována a že myší vajíčka reprezentují zvláštní buněčný typ, ve kterém je dvouvláknová RNA cílena do RNA interferenční dráhy.

Pokusy založené na tranzientní transfekci dále ukázaly, že plazmidy produkující dvouvláknovou RNA tlumí expresi společně transfekovaných reportérových plazmidů, ale pouze minimálně působí na expresi endogenních genů nebo reportérů stabilně začleněných do genomu. K této inhibici pravděpodobně dochází na úrovni iniciace translace a je zprostředkována místní a přechodnou aktivací protein kinázy R. Naše výsledky naznačují, že buňka by mohla být schopna odlišit endogenní mRNA od exogenní a v přítomnosti dvouvláknové RNA tlumit translaci cizí mRNA.

Analýza transkriptomu buněk transfekovaných běžně používanými plazmidy ukázala, že transkripce plazmidů je komplexní a může způsobit vznik dvouvláknové RNA párováním komplementárních úseků transkriptů. Naše výsledky ukazují, že vznik dvouvláknové RNA nelze předpovídat a že přítomnost dvouvláknové RNA v buňce může vést ke špatné interpretaci dat založených na tranzientních transfekcích.

Abstract

Double stranded RNA (dsRNA) is a foreign molecule that arises in the cell either as a by-product of viral replication or it is produced by the intramolecular or intermolecular pairing of complementary cellular RNAs, often originating from repetitive sequences. In mammals, dsRNA can enter one of three pathways: the sequence-specific RNA silencing, the sequence-independent interferon (IFN) response, or editing by adenosine deaminases.

The main focus of my PhD project was to comprehensively analyze the effects of the expressed dsRNA in mammals in the context of the whole organism. To follow this aim, we generated a construct expressing dsRNA in a form of an mRNA containing a long perfect hairpin structure.

Transgenic mice ubiquitously expressing dsRNA were viable and, in contrast to the previous studies, the IFN response was not activated. In somatic cells, dsRNA was poorly processed into small interfering RNAs, did not cause transcriptional silencing *in trans*, and underwent low adenosine deamination without the nuclear retention. Similar results were obtained in human cells transiently transfected with a dsRNA-expressing plasmid. On the other hand, dsRNA expression caused robust RNA interference (RNAi) in oocytes. Thus, we show for the first time that expressed dsRNA, in contrast to many other forms of dsRNA, can be well tolerated in mammals and that oocytes represent a privileged cell type in terms of directing dsRNA into the RNAi pathway.

In addition, transient transfection experiments revealed that dsRNA-expressing plasmids inhibit expression of co-transfected reporter plasmids, but they have a minimal impact on the expression of endogenous genes or reporters stably integrated in the genome. The inhibition likely occurs at the level of translation initiation and it is mediated by the local and transient activation of protein kinase R. Accordingly, our results indicate that cells might be able to distinguish between endogenous and exogenous mRNAs and selectively inhibit the translation of foreign mRNAs in response to dsRNA.

Finally, the transcriptome analysis of cells transfected with commonly used plasmids showed that plasmid transcription is complex and may result in the dsRNA formation via basepairing of complementary sense and antisense transcripts. Our results demonstrate that the formation of dsRNA structure cannot be efficiently predicted and can cause misinterpretation of data based on transient transfection experiments.

1. Introduction

Double-stranded RNA (dsRNA) is a double-helical molecule formed by pairing of two antiparallel RNA strands. Its sources can be either exogenous (viral replication) or endogenous (intramolecular or intermolecular pairing of complementary sequences). The internal sources of dsRNA generally come from the activity of transposable elements, repetitive sequences, genome inversions, small RNA precursors, or overlapping transcripts [1].

Depending on an origin, dsRNA can be found either in the nucleus or in the cytoplasm. Cellular localization represents one of the key features that affect dsRNA fate, mainly due to the availability of dsRNA-binding proteins (DRBP). DRBPs typically contain one or more dsRNA-binding domains, which recognize dsRNA according to its A-form conformation; thus, DRBPs usually bind dsRNA without a sequence-specificity [2].

In mammals, dsRNA recognition usually elicits one of three dsRNA-responding pathways: RNA interference (RNAi), interferon (IFN) response, or ADAR editing (reviewed in [3]).

1.1. RNA interference

RNAi is a member of RNA silencing pathways (together with microRNA and PIWI-interacting pathways). RNAi is conserved in almost all eukaryotes and mediates a sequence-specific degradation of RNAs complementary to dsRNA (reviewed in [4]). In RNAi, long dsRNA substrate is processed by the RNase III Dicer into small interfering RNAs (siRNAs), 21-22 nucleotide (nt)-long molecules with a 5’ monophosphate group and a 3’ dinucleotide overhang. One of the siRNA strands is incorporated into the RNA-induced silencing complex (RISC) and acts as

a guide for binding to the complementary mRNA. The cognate mRNA is subsequently cleaved in the middle of the basepairing region by AGO2 protein and rapidly degraded.

1.2. Interferon response

The origin of dsRNA as a by-product of viral replication makes it a convenient target for the innate immune response machinery. Indeed, dsRNA is recognized by a handful of proteins collectively operating in the complex of pathways whose common aim is to signal infection, restrict viral growth, and limit viral spread [5]. As the hallmark of this pathway is a production of antiviral cytokines IFNs, the response was termed the IFN response. In contrast to RNAi, the IFN response is limited to vertebrates and it generally recognizes dsRNA molecule without any concern to its sequence. Two branches form the backbone of the IFN response: protein kinase R (PKR) and 2'5'-oligoadenylate synthetase (OAS)/RNase L systems.

PKR alias 'IFN-inducible protein kinase' or 'eukaryotic translation initiation factor 2-alpha kinase 2' (EIF2αK2) is a cytoplasmic serine/threonine kinase. It contains a dsRNA-binding domain and a kinase domain. PKR can be activated by several molecules, mainly dsRNA of viral, cellular, or synthetic origin or stress signals associated with the viral infection. PKR activation leads to the phosphorylation of its substrates; the main substrate is alpha subunit of eukaryotic initiation factor 2 (eIF2α). Phosphorylated eIF2α is responsible for the inhibition of translation initiation, resulting in a blockage of cellular proteosynthesis, inhibition of viral spread, and eventually apoptosis [6]. Although PKR activation is known to inhibit cellular translation globally, several reports demonstrated the selective inhibition of plasmid-derived proteosynthesis without affecting the expression of endogenous genes [7, 8]. Moreover, except its

prevalent cytoplasmic localization, PKR was also detected in nuclei [9] or associated with ribosomes [10], suggesting that the role for PKR is not fully elucidated, yet.

The second part of the IFN response is represented by OAS/RNase L system. OAS is a protein that binds to dsRNA, but it does not contain a dsRNA-binding domain. Upon binding dsRNA, OAS is activated and converts adenosine triphosphates to pyrophosphates and 2'5'-linked oligoadenylates that activate RNase L. Activated RNase L degrades non-specifically all viral and cellular RNAs, resulting in a decreased proteosynthesis [11].

In addition, the IFN response involves additional molecules, such as retinoic acid-inducible gene 1, melanoma differentiation-associated gene-5, toll-like receptors, IFN-stimulated gene 15, or Mx GTPases [5, 12].

1.3. RNA editing

Besides RNA silencing and the IFN response, dsRNA serves as a substrate for 'adenosine deaminase acting on RNA' (ADAR) enzymes. One to three ADARs are present in all animals; in contrast, they are completely absent in protozoa, yeasts, and plants. ADARs recognize largely or completely double-stranded RNAs in the size over 20 bp (two turns of the dsRNA helix). In these substrates, adenosine is deaminated to inosine (a nucleoside in which hypoxanthine is attached to ribose). Newly-arisen inosine is recognized as guanosine by the translation and splicing machineries; hence, ADAR editing can influence mRNA processing and protein coding. In addition, inosine preferentially pairs with cytidine (instead of original adenosine – uridine pair), which changes RNA structure (reviewed in [13, 14]).

For short dsRNAs (20-30 bp) or dsRNAs containing bulges, mismatches, or loops, ADAR editing is usually selective (hypoediting). In contrast, long (>100 bp)

and perfect substrates are edited randomly and massively, with up to 50 % of all adenosines converted (hyperediting); hyperedited RNAs are often captured in the nucleus and degraded (reviewed in [14, 15]).

1.4. Interaction among dsRNA-activated pathways

dsRNA is a substrate for several enzymes, mediating different cellular functions; hence, the selection of a particular dsRNA processing enzyme depends on various factors. Accordingly, different dsRNA-binding proteins process different types of dsRNA, with respect to dsRNA length, termini, or mismatches. In addition, some pathways are present only in some organisms, tissues, or subcellular localization (summarized in [4, 5]).

1.5. dsRNA-activated pathways in oocytes

Mammalian oocytes respond to dsRNA presence much differently than somatic cells. The IFN response and miRNA pathways are not functional in mouse oocytes; in contrast, RNAi is much more efficient in mouse oocytes compared to somatic cells [16]. Therefore, mouse oocytes represent a special cell type where the sequence-specific effect of long dsRNA can be studied without the risk of non-specific effects caused by the IFN response.

1.6. Background of the project

This project studying dsRNA-activated pathways in mammals was based on preceding results from Richard Schultz group, studying the effects of long dsRNA

(with a *Mos* gene sequence) in mouse oocytes [17-20]. The previous data demonstrated that long dsRNA induces a sequence-specific degradation of complementary mRNA in mouse oocytes [17], without inducing off-target effects or the IFN response [20]. In addition, they showed that the IFN response is suppressed in mouse oocytes [20]. This suggested that RNAi mechanism may be functional in mammals and that mouse oocytes represent a special cell type.

Moreover, the Schultz group generated a model system utilizing a transgene expressing a long inverted repeat of the mouse *Mos* gene sequence [18, 19], which was adopted in this project.

2. Aims of the project

The main objective of this PhD project was to explore how mammals respond to long dsRNA expression. This study provides a comprehensive view on the effects of dsRNA (1) in the context of the whole organism (mice expressing dsRNA) and (2) under a variety of cell culture conditions. In contrast to previously used dsRNA model systems, we utilized dsRNA expressed in the nucleus in the form of an mRNA with a long hairpin structure.

We aimed to address following objectives:

- Develop a model system that generates dsRNA *in vivo*
- Determine if dsRNA expression can be tolerated in mammals
- Analyze which pathways are recruited for MosIR processing
- Discover how dsRNA-responding pathways intersect
- Monitor how the response to dsRNA differs across different cell types
- Examine the behavior of different dsRNA types and origins

The revelation that expressed dsRNA inhibits proteosynthesis of co-transfected reporters without affecting cellular viability established additional aims:

- Investigate the mechanism of the reporter inhibition
- Examine the fate of transfected plasmids
- Reveal how cells distinguish between endogenous and exogenous mRNAs

3. Overview of materials and methods

Transgenic mice:

Three transgenic mouse strains were used in this project: MosIR (expressing Mos hairpin), Mos3 (expressing Mos target sequence in the 3'UTR of the transgene), and MosP (expressing Mos target sequence in the promoter).

The phenotype analysis of transgenic mice included isolation of oocytes, somatic tissues, and mouse embryonic fibroblasts.

Cell culture:

Human cell lines (HeLa and HEK293) were used for transfections and for generation of stable lines with PKR knock-down and stable expression of *Renilla* luciferase, firefly luciferase, or both reporters.

Analysis of dsRNA-expressing mice and cell lines

Transcriptome changes were assessed by RNA isolation, followed by reverse transcription real-time PCR, microarray analysis, or next-generation sequencing (SOLiD). Changes at protein level were analyzed by luciferase assay, immunofluorescence microscopy, and western blotting.

RNA editing was monitored by sequencing of cloned RT-PCR products or next-generation sequencing.

The ability of MosIR to fold into dsRNA was verified by the digestion of single-stranded RNA by RNase T1 and real-time PCR.

Analysis of spurious plasmid transcription and dsRNA-expression effects

Stably or transiently transfected cells were analyzed by real-time PCR, next generation-sequencing, western blotting, and flow cytometry.

The mechanism of selective translation inhibition was studied using polysome profiling and RNA immunoprecipitation.

4. Results and discussion

4.1. Model system for dsRNA expression

dsRNA was expressed from the transgene (pCagEGFP-MosIR), containing an insert of Mos inverted repeat (MosIR) in the 3' untranslated region of enhanced green fluorescence protein expressed from a ubiquitous polymerase II promoter. Upon transcription, the MosIR presumably folds into a perfect hairpin (~500 bp) with a short loop; thereby, the resulting dsRNA is embedded in a spliced, capped, and polyadenylated mRNA [16]. The ability of the construct to efficiently fold into dsRNA was verified using several independent approaches [16].

4.2. Effects of long dsRNA expression in the mouse and cell culture

The main part of the project studying long dsRNA expression in mammals analyzed the phenotype of transgenic mice ubiquitously expressing Mos hairpin RNA. MosIR mice were viable without any defects; therefore, our transgene represents the first model showing that ubiquitous dsRNA expression can be well tolerated in mammals [16].

Although the transgene was expressed in all tested tissues and MosIR efficiently formed dsRNA, the loading into dsRNA-activated pathways differed between somatic cells and oocytes. The response to MosIR in oocytes mimicked the phenotype of Mos knock-out mice [21], providing additional evidence that mouse oocytes possess efficient RNAi [16]. In contrast, RNAi effects were negligible in somatic cells. Although we detected MosIR-derived siRNAs, their abundance was too low to induce efficient RNAi [16], presumably due to less

processive isoform of Dicer in somatic cells compared to oocytes (Flemr *et al.*, submitted).

In our dsRNA-expressing model system, we did not find any evidence for the activation of the IFN response. The analysis of both transgenic mice and cell lines transfected with MosIR-expressing plasmid did not exhibit marked transcriptome changes, including changes in expression of IFN-stimulated genes [16]. On the other hand, dsRNA derived from MosIR underwent low but clearly detectable adenosine deamination, but the editing did not likely interfere with nuclear export and translation [16].

The explanation for the lack of the IFN response in our system, which differs from a great deal of other studies (summarized in [3]), plausibly lies in the nature of dsRNA used in our model system. There are several types of dsRNA (differing in the length, structure, termini, *etc.*) that likely determine which response is activated. In addition, the dose, subcellular localization of dsRNA, its delivery into the cell, and the cell type used for the study creates another set of factors influencing the activation of the IFN response and other dsRNA-activated pathways (reviewed in [3]). Accordingly, our dsRNA expressed in the nucleus in the form of a capped, polyadenylated, and spliced mRNA seems not to be recognized as deleterious; at the same time, it preserves the dsRNA structure necessary for its recognition by other dsRNA-activated pathways. The ability of cells to tolerate expressed dsRNA may have an important evolutionary role. As there is a widespread overlapping transcription in the genome (coming from repetitive sequences, activity of transposable elements, or regulatory antisense RNAs), it is vital to tightly regulate whether and which response to dsRNA will be triggered.

4.3. Effects of dsRNA-expressing plasmids on co-transfected plasmids

The sequence-specific and sequence-independent effects of dsRNA expression can be monitored using co-transfected reporter plasmids, such as luciferase-expressing plasmids. A transient co-transfection of dsRNA-expressing plasmid together with luciferase-expressing reporters revealed a concentration-dependent decrease in luciferase activities. This inhibition was independent of a cell type, transfection method, or sequence of dsRNA-expressing plasmid. On the other hand, the effect required the presence of a transcribed inverted repeat. Further experiments showed that the inhibition of co-transfected reporters occurs post-transcriptionally, plausibly at the level of translation initiation, and it involves PKR activity. Remarkably, dsRNA-expressing plasmids did not affect endogenous genes (Nejepinska *et al.*, manuscript attached to the thesis).

The selective translation inhibition of transiently co-transfected plasmids but not genome-integrated genes mediated by PKR indicates that there can be an alternative pathway involving PKR upregulation and phosphorylation but without the activation of a classical IFN response and general block of proteosynthesis.

4.4. Transcription of transiently transfected plasmids

The analysis of RNA transcriptome of transfected plasmids by massive parallel sequencing revealed that RNA is produced from the entire plasmid, including the plasmid backbone, antisense strand of the coding region, and the intron. Although the annotated eukaryotic transcription units yielded the highest read densities, significant amounts of RNA were generated from regions that are not expected to be transcribed in mammalian cells ([22] and Nejepinska *et al.*, manuscript attached to the thesis).

This spurious transcription can form dsRNA and affect results of transient co-transfection experiments, as exemplified by the inhibition of luciferase activities caused by plasmids expressing the kanamycin/neomycin resistance cassette [22]. Accordingly, we propose the mechanism of the reporter inhibition by formation of dsRNA from convergent transcription originating either in kanamycin/neomycin resistance region [22] or hairpin-expressing plasmids (Nejepinska *et al.*, manuscript attached to the thesis).

As transient co-transfection represents a common tool, a detailed understanding of the fate of plasmids in cells is crucial for a wide spectrum of scientists. Therefore, our results highlight the need to include proper controls and to carefully choose an extra DNA that is used to equalize the total amount of transfected DNA.

5. Conclusions

Long dsRNA triggers RNAi in plants and invertebrates, while it is a potent inducer of the antiviral IFN response in vertebrates. However, no integrated study of the effects of the expressed dsRNA in mammals has been conducted, yet. Most reports focus either on sequence-independent inhibitory effects of dsRNA or they utilize dsRNA for RNAi studies in cell types where the sequence-specific RNAi is functional. Moreover, the majority of studies employ *in vitro*-transcribed dsRNA that is more immunostimulatory than expressed dsRNA used in this project. Therefore, this work represents a comprehensive study of dsRNA effects in mammals, demonstrating that dsRNA (expressed either in transgenic mice or in transfected cultured cells) can be well tolerated without provoking the IFN response. At the same time, expressed dsRNA retains features necessary for the processing into siRNAs by Dicer or editing by ADARs. Accordingly, it seems that the limiting factor for RNAi in mammalian somatic cells is inneficient Dicer processing.

In addition, this work demonstrates that transcription of transfected plasmids is not restricted to defined transcription units, but it involves all plasmid regions. This spurious convergent transcription can lead to dsRNA formation and can suppress translation of co-transfected reporter plasmids, leading to misinterpretation of results. Many commonly used plasmids have a potential to generate dsRNA; however, dsRNA formation cannot be efficiently predicted. As transient transfection is a widely used method, our report documenting the inhibition of co-transfected plasmids indicates that further studies of dsRNA are highly desirable.

The follow-up research needs to address which factors are responsible for different RNAi efficiency between somatic cells and oocytes and explore in more detail how cells distinguish between endogenous and exogenous DNA and RNA.

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List of publications

Publications related to this thesis:

Nejepinska J, Malik R, Filkowski J, Flemr M, Filipowicz W, Svoboda P. dsRNA expression in the mouse elicits RNAi in oocytes and low adenosine deamination in somatic cells. Nucleic Acids Res. 2012 Jan;40(1):399-413. (IF = 8.026)

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Chalupnikova K, Nejepinska J, Svoboda P. Production and application of long dsRNA in mammalian cells. Methods Mol Biol. 2013;942:291-314. (Book chapter)

Nejepinska J, Flemr, Svoboda P. The canonical RNA interference pathway in animals. Regulatory RNAs. 2012;111-150. (Book chapter)

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Nejepinska J, Malik R, Svoboda P. Expressed ds RNA provokes selective sequence-independent PKR response in transient co-transfection experiments. (Manuscript attached to the thesis)

Flemr M, Malik R, Franke V, Nejepinska J, Beck IM, Vlahovicek K, Svoboda P. A retrotransposon-driven variant of mouse Dicer fuels endogenous siRNA production. (Submitted)

Other publications:

Nejepinska J, Juklova K, Jirsova K. Organ culture, but not hypothermic storage, facilitates the repair of the corneal endothelium following mechanical damage. Acta Ophthalmol. 2010 Jun;88(4):413-9. (IF=2.629)

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