KAUNAS UNIVERSITY OF TECHNOLOGY

EGLĖ JAKUBAUSKIENĖ

PRE-mRNA SPLICING: REGULATION OF MONOAMINE OXIDASE B AND OXYGEN-DEPENDENT GENES EXPRESSION

Summary of Doctoral Dissertation
Technological Sciences, Chemical Engineering (05T)

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KAUNO TECHNOLOGIJOS UNIVERSITETAS

EGLĖ JAKUBAUSKIENĖ

PRE-iRNR SPLAISINGAS: MONOAMINOOKSIDAZĖS B IR NUO DEGUONIES KIEKIO PRIKLAUSOMŲ GENŲ RAIŠKOS REGULIACIJA

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Introduction

Relevance of the Work

Special attention to human diseases has been increasing during the recent decades; therefore, there has been a systematically growing demand for more exhaustive information about the alterations of biological processes inside the cell. It has been acknowledged that numerous human diseases can be caused by the disruption of the normal pre-mRNA splicing patterns.

Pre-mRNA splicing takes places in the nucleus. It consists of the removal of non-coding sequences (introns) from mRNA precursors (pre-mRNAs) and joining of coding sequences (exons) to generate the mature mRNA. The inaccuracies of pre-mRNA splicing process play an important role in aberrant mRNA isoform formation and changes to gene expression profiles which are associated with neurodegenerative, pulmonary, cardiovascular diseases and cancer (Douglas & Wood, 2011; Faustino & Cooper, 2003). Therefore, studies of mutation causes in pre-mRNA sequence, their influence upon the mRNA formation rate or the changes in the splicing factor activity as well as their effects towards the alternative splice site choice on the pre-mRNA promoting different mRNA isoform formation are very important. The work presented in this thesis increases our knowledge about the reasons of alterations in pre-mRNA as one of the factors in the development of human diseases.

This study is focused on the influence of pre-mRNA splicing to the regulation of monoamine oxidase B (MAO-B) mRNA formation and the identification of splicing factors involved in oxygen tension-dependent alternative pre-mRNA splicing regulation.

Parkinson's disease (PD) belongs to a chronic neurodegenerative disorder affecting people over the age of 50–70 and is associated with the changes in the brain area called *substantia nigra* which controls voluntary movements. A single nucleotide polymorphism (G changes to A) which is found in the non-coding sequence of the *MAO-B* gene is related to the development of Parkinson's disease (Balciuniene *et al*, 2002; Dorszewska *et al*, 2013). Our study demonstrates for the first time that this polymorphism affects the efficiency of the intronic sequence removal from MAO-B pre-mRNA and that it influences protein expression levels in cells. The expression of MAO-B protein level increases in the blood platelet fraction of patients with Parkinson's disease compared to healthy individuals and could serve as a Parkinson's disease marker. Such findings provided novel information about G/A polymorphism in the non-coding sequence and its influence on MAO-B mRNA formation and consequently on the pathogenesis of Parkinson's disease.

An extensive range of various diseases has been associated to the decreased cellular oxygen level, including ischemic, peripheral and coronary artery diseases, pulmonary hypertension, anemia, obstructive sleep apnea,

erythrocytosis and cancer. The reduction of oxygen levels in cellular microenvironment (hypoxia) induces the stabilization of hypoxia-inducible transcription factors (HIFs) which activate transcription of genes involved in the ability of cells to adapt to the altered conditions (Semenza, 2014). Alternative pre-mRNA splicing also plays an important role in cellular response to hypoxia producing multiple mRNA isoforms from the same pre-mRNA (Khan *et al*, 2012; Sena *et al*, 2014). We demonstrate that hypoxic conditions change the alternative splice site usage on pre-mRNA by changing the activity of the essential splicing factors called SR proteins. The results from these studies provide a more general mechanism in hypoxia-dependent gene expression regulation.

Aim

Part I. Relation between a Single Nucleotide Polymorphism in Intron 13 Sequence of the *MAO-B* Gene and pre-mRNA Splicing

To analyze the effect of G/A polymorphism in *MAO-B* gene intron 13 sequence for pre-mRNA splicing, to identify the factors involved in such regulation and to study the MAO-B protein expression levels in the platelet fraction of healthy individuals and Parkinsonian patients.

Part II. Hypoxia-Dependent Alternative pre-mRNA Splicing Regulation in Eukaryotic Cells

To identify factors regulating oxygen tension-dependent alternative pre-mRNA splicing and to find a possibility to control the hypoxia-dependent splice site usage.

Specific Tasks of the Dissertation

Part I.

1. To compare the MAO-B protein expression levels in Parkinsonian patients versus healthy individuals and to elucidate whether MAO-B could serve as a disease marker.
2. To study G/A polymorphism in *MAO-B* gene intron 13 influence for pre-mRNA splicing.
3. To identify factors influencing MAO-B pre-mRNA splicing.

Part II.

1. To identify factors regulating oxygen tension-dependent pre-mRNA splicing.
2. To investigate how cellular hypoxia conditions influence the splicing factors regulating hypoxia-dependent splicing.

3. To evaluate whether the results obtained from pre-mRNA splicing experiments *in vitro* correspond to the results from endogenous alternatively spliced hypoxia-dependent pre-mRNA in cells.

Scientific Novelty and Practical Importance of the Thesis

The present investigation consists of two parts: Part One covers the influence of mutation in pre-mRNA for constitutive pre-mRNA splicing; Part Two deals with the regulation of alternative pre-mRNA splicing which is dependable on the oxygen levels in cellular micro environment and with the association of changes in pre-mRNA splicing with human diseases. The research was carried out at Vilnius University, Institute of Biotechnology, Department of Immunology and Cell Biology.

The first part of the work presents a detailed study of how a single nucleotide polymorphism in *MAO-B* gene non-coding sequence may influence pre-mRNA splicing. For the first time, we show that G/A polymorphism has an important role in intron 13 removal efficiency. The removal of this non-coding sequence from pre-mRNA might be a rate limiting factor in the MAO-B mRNA formation. We have established that SR proteins interact more efficiently with RNA sequence containing the "A" allele compared to a sequence containing the "G" allele. Also, we have shown that differential SR protein interaction with RNA effects the formation of the early spliceosomal complex. The results we have obtained might potentially explain the reason of enhanced MAO-B mRNA and protein expression levels in patients with Parkinson's disease. This data indicates that the increased MAO-B protein levels might be used as a marker for Parkinson's disease.

In the second part of our study, the factors involved in hypoxia-dependent alternative pre-mRNA splicing regulation were elucidated. We used mice HIF-3α pre-mRNA as a model system for *in vitro* splicing assays in nuclear extracts prepared from HeLa cells cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions. Detailed studies revealed that SR proteins isolated from hypoxic cells possess differential ability in pre-mRNA splicing activation compared to proteins isolated from cells cultured under normoxic conditions. For the first time we report that SR proteins in hypoxic cells are hyper-phosphorylated in comparison with normoxic cells, and this enhancement is caused by an increase in the expression of specific SR protein kinases (SRPK1, SRPK2 and CLK1) in hypoxic cells. We have thus shown that an increase in the expression of CLK1 kinase in hypoxic cells is regulated by hypoxia-inducible factor HIF-1. The obtained results in an *in vitro* system were confirmed by experiments in cells where endogenous hypoxia-dependent pre-mRNAs splicing was analyzed. Taken together, these findings can be useful for our understanding of a more general mechanism of hypoxia-dependent gene expression regulation. The possibility of

reduction of aberrant mRNAs formation in hypoxic cells is very important for the prevention of development of human hypoxia-associated diseases.

Defended Statements of the Scientific Work

Part I.

1. MAO-B protein expression levels in platelets of Parkinsonian and healthy patients could serve as a disease marker.
2. G/A polymorphism in intron 13 sequence effects MAO-B pre-mRNA splicing.
3. SR proteins are one of MAO-B expression regulators.

Part II.

1. Reduced levels of cellular oxygen tension change the activities of essential splicing factors – SR proteins.
2. Changes in SR protein activity in hypoxic cells determine differential protein RS domain phosphorylation levels.
3. Increased in SR protein phosphorylation levels in hypoxic cells are determined by the increased expression of specific SR protein kinases.
4. Reduction of CLK1 expression in hypoxic cells promotes usage of normoxic splice sites.

Materials and Methods

Platelet fraction was prepared from healthy individuals' and Parkinsonian patients' blood and analyzed by performing Western blot analysis. HeLa cells cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions were used for nuclear extracts preparation and isolation of SR proteins.

MAO-B, mouse HIF-3α, IPAS, rabbit β-globin, and chimeric β-globin-MAO-B DNA constructs were generated for *in vitro* transcription reaction and splicing assays. Spliceosome early complex assembly was analyzed. In order to investigate the RNA – protein interaction, the UV crosslinking method was used. When seeking to determine differentially with RNA interacting proteins and cellular protein expression, the Western blot method was employed.

Also, cDNAs synthesis and RT-PCR (reverse transcription – PCR) reactions were used for endogenous pre-mRNA splicing studies. Protein modifications were analyzed by performing two-dimensional gel electrophoresis. Chromatin immunoprecipitation (ChIP) and real-time PCR (qPCR) assays were done in order to determine the reason of the enhanced expression of kinase. The reduction of protein expression at the cellular level was performed by treatment with the appropriate siRNA, and specific inhibitors were used when seeking to reduce the enzymatic activity.

Results and Discussion

Pre-mRNA splicing is one of the essential steps in the regulation of the eukaryotic gene expression, and it is not surprising that the disruption of the normal splicing patterns can lead to various human diseases or contribute to their severity (Cooper *et al*, 2009; Douglas & Wood, 2011).

Part I. Relation between a Single Nucleotide Polymorphism in Intron 13 Sequence of the *MAO-B* Gene and pre-mRNA Splicing

Monoamine oxidase B (MAO-B) has an important role in the metabolism of monoamines in the central nervous system and peripheral tissues (Nagatsu & Sawada, 2006; Nikolac Perkovic *et al*, 2016).

It has been shown that MAO-B mRNA levels and enzymatic activity increase in the platelet fraction of patients with Parkinson's disease due to some unknown reason (Balciuniene *et al*, 2002). We have studied the expression of MAO-B protein in the platelet fraction obtained from the blood of healthy individuals and Parkinsonian patients. The increased levels of MAO-B protein expression have been found in PD patients when compared to healthy persons (Figures 1A and 1B). The isolated platelet DNA sequencing data from patients with PD showed the association of the "A" allele with the enhanced MAO-B expression levels.

In order to study the effect of G/A polymorphism in *MAO-B* gene for pre-mRNA splicing, we have generated constructs containing "G" or "A" alleles in intronic sequence (MAO-B_g and MAO-B_a, respectively) (Figure 1C). These constructs were tested in *in vitro* splicing assays, and the results demonstrated the enhanced efficiency of MAO-B_a pre-mRNA splicing compared to MAO-B_g construct splicing (Figures 1D and 1E). It means that the intron 13 containing the "A" allele is removed from pre-mRNA more efficiently than the "G" allele.

In order to elucidate whether one nucleotide change in intron 13 is sufficient to influence the pre-mRNA splicing efficiency, we have generated chimeric β-globin constructs containing "G" or "A" alleles in intronic MAO-B sequences (β-gl-MAO-B_g and β-gl-MAO-B_a, respectively) (Figure 2A). These chimeric constructs were used in *in vitro* splicing assays. The obtained results showed that the β-gl-MAO-B_a construct was spliced more efficiently than the β-gl-MAO-B_g construct (Figures 2B and 2C). This indicates that one nucleotide-related changes in the intronic sequence may greatly influence the intron removal efficiency.

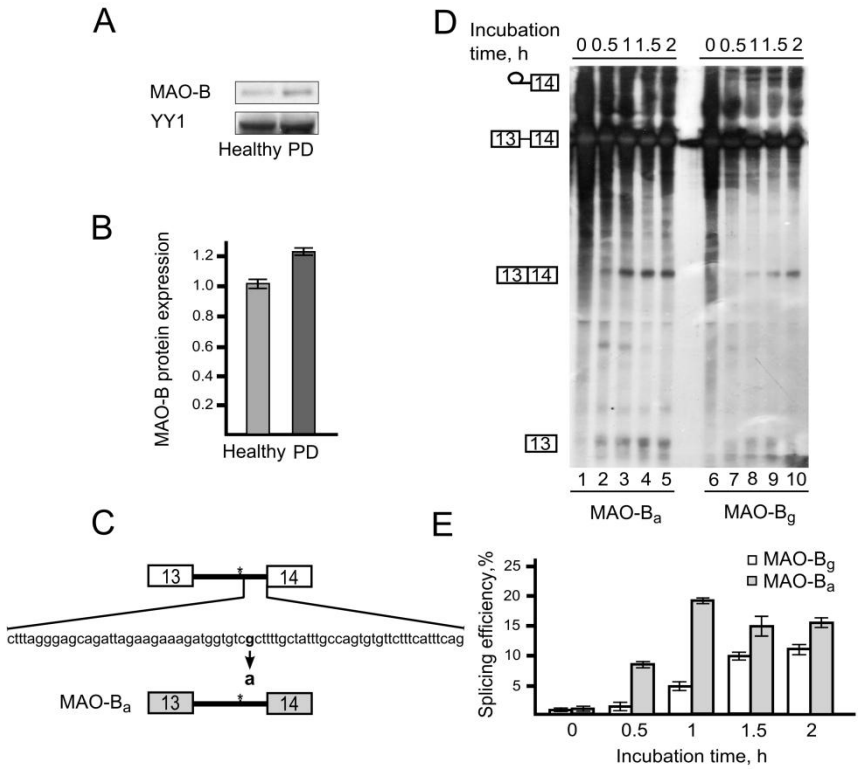

Figure 1. MAO-B expression levels and the effect of the "A" allele for MAO-B intron 13 splicing. (A) MAO-B protein expression levels in the platelet fraction from one healthy person and one patient with Parkinson's disease. YY1 – loading control. (B) Quantitation of MAO-B protein expression levels in the platelets from PD patients was normalized against the expression levels of healthy individuals (n = 8) by using scanning imager platform (Fuji FLA-5100 imaging system) ($p < 0.01$). (C) Schematic representation of pre-mRNA constructs used in the *in vitro* splicing assays. MAO-B_g contains the "G" allele, whereas MAO-B_a contains the "A" allele. The enlargement shows the nucleotide sequence, the G/A polymorphism is indicated with an arrow. The branch point is indicated by an asterisk (*). (D) Time course (0–2h) of MAO-B_g (lanes 1–5) and MAO-B_a (lanes 6–10) transcripts in *in vitro* splicing assays. Lanes 1 and 6 feature splicing reactions incubated at 0h, lanes 2 and 7 – at 0.5h, lanes 3 and 8 – at 1h, lanes 4 and 6 – at1.5h, lanes 5 and 10 – at 2h. (E) Calculation of spliced product formation (data from three independent experiments; $p < 0.05$) obtained by using a scanning imager platform (Fuji FLA-5100 imaging system)

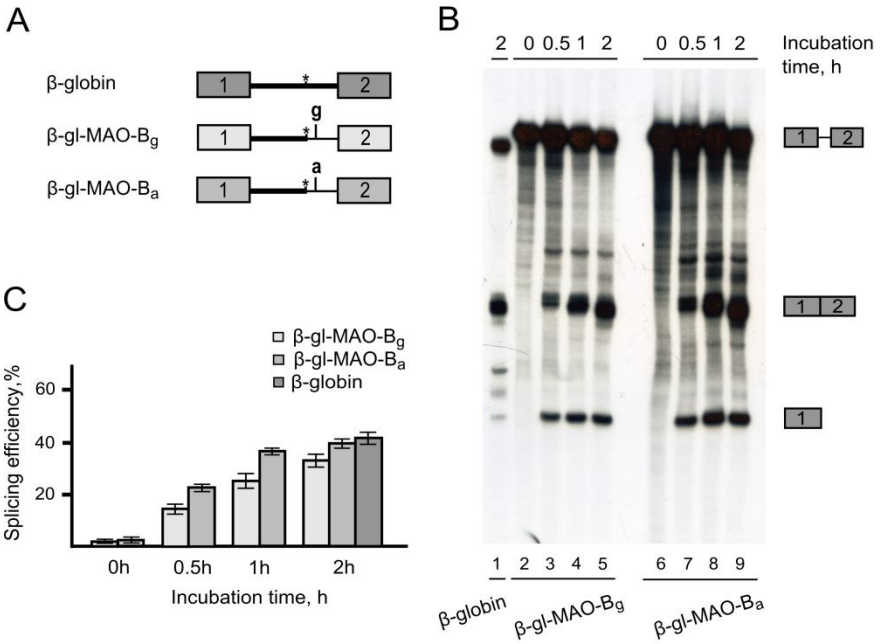

Figure 2. Effect of G/A polymorphism in the intron sequence for chimeric pre-mRNAs splicing. (A) Schematic representation of chimeric constructs used in *in vitro* splicing assays. β-gl-MAO-B_g contains the "G" allele, β-gl-MAO-B_a contains the "A" allele. The branch point is indicated by an asterisk (*). (B) Time course (0–2h) of β-globin (lane 1), β-gl-MAO-B_g (lanes 2–5) and β-gl-MAO-B_a (lanes 6–9) chimeric construct splicing reaction *in vitro*. Lanes 2 and 6 splicing reactions incubated for 0h, lanes 3 and 7 – for 0.5h, lanes 4 and 8 – for 1h, lanes 1, 5 and 9 – for 2h. (C) Calculation of spliced product formation (data from three independent experiments, $p < 0.05$) using a scanning imager platform (Fuji FLA-5100 imaging system)

The G/A polymorphism in *MAO-B* gene intron 13 is located near the branch point sequence. In order to test whether this polymorphism affects the branch point location during spliceosome formation, we used bioinformatics analysis (Desmet *et al*, 2009). The obtained data shows that changes from G nucleotide to A nucleotide in *MAO-B* gene intronic sequence do not affect the branch point location.

Subsequently, we have studied at which stage of the spliceosome formation the efficiency of the intron removal is affected. Spliceosome assembly experiments demonstrated that the early stage of spliceosome complex formation was more efficient on the MAO-B_a pre-mRNA transcript compared to the MAO-B_g transcript (Figure 3A). The obtained results indicated that A nucleotide in *MAO-B* gene non-coding sequence increases the efficiency of the spliceosome A

complex assembly. As a next step, we have examined how proteins from the nuclear extracts of HeLa cells interact with MAO-B_g and MAO-B_a pre-mRNA transcripts. UV crosslinking analysis showed that proteins from nuclear extracts interacted with MAO-B_a pre-mRNA more efficiently when compared to MAO-B_g (Figure 3B). In order to identify the splicing factors differentially interacting with pre-mRNAs, we have crosslinked proteins from HeLa nuclear extract to MAO-B_g and MAO-B_a pre-mRNAs by using UV radiation. Proteins crosslinked to pre-mRNAs were "fished out" from nuclear extracts using streptavidin beads and analyzed by performing Western blot analysis. Immunoblot with the anti-SR antibody showed that SR proteins interacted more efficiently with the construct containing an *A* nucleotide when compared to a *G* nucleotide (Figure 3C). These findings suggest that the G nucleotide changes to the A nucleotide in intron 13 creating an SR protein binding site and thus promoting the spliceosomal complex formation.

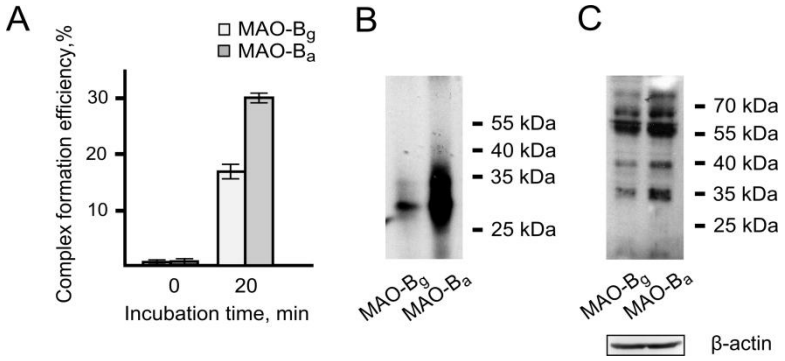

Figure 3. Effect of the "A" allele for an early stage spliceosome assembly formation and interaction with splicing factors. (A) Quantification results (from three independent experiments; $p < 0.03$) of splicesomal assembly on MAO-B_g and MAO-B_a pre-mRNAs. (B) Radiogram of UV crosslinked proteins from HeLa cell nuclear extracts with [^{32}P]-CTP labeled MAO-B_g and MAO-B_a transcripts. (C) Western blot analysis of proteins with anti-SR antibody fished out from nuclear extracts with streptavidin beads bound to double labeled ([^{32}P-CTP] and biotin) MAO-B_g and MAO-B_a pre-mRNAs. β-actin was used as the loading control

The addition of isolated SR proteins from HeLa cells (Figure 4B) into splicing reactions revealed that MAO-B_g constructs require much higher SR protein concentration for splicing activation in comparison with MAO-B_a constructs (Figures 4A and 4C). These experiments indicate that intron containing the "A" allele increases the SR protein binding efficiency by stimulating the intron removal efficiency.

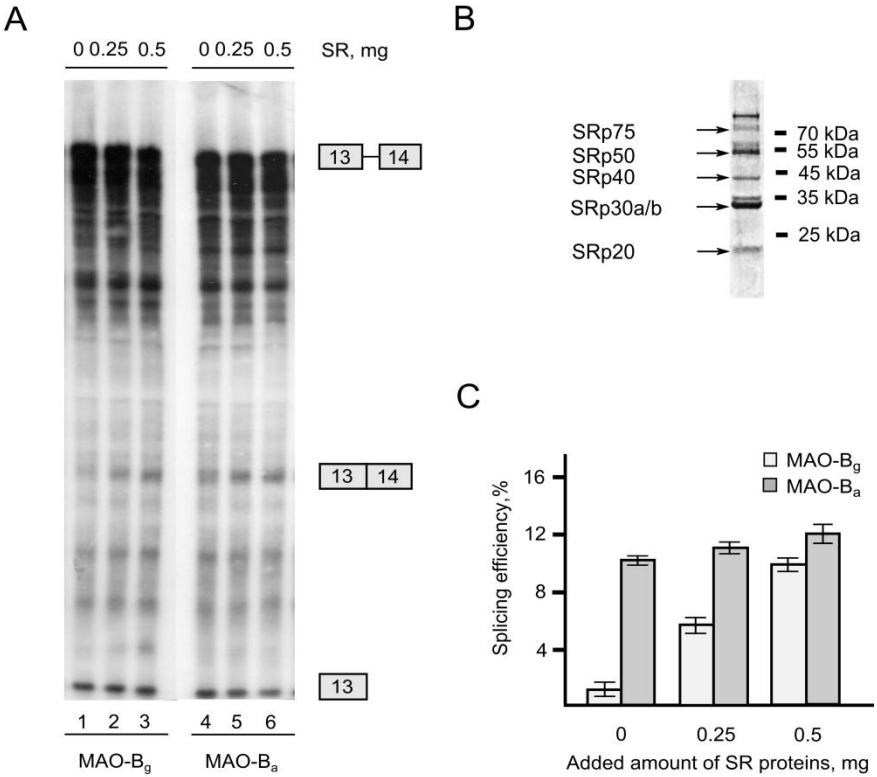

Figure 4. SR proteins activate MAO-B_g and MAO-B_a constructs splicing differentially. (A) Influence of purified SR proteins on MAO-B_g (lanes 1–3) and MAO-B_a (lanes 4–6) pre-mRNA splicing. Lanes 2 & 3 and 4 & 5 feature increasing amounts (0.25 and 0.5 μg) of SR proteins, respectively. (B) SDS-PAGE of SR proteins isolated from HeLa cells. (C) Average of MAO-B_g and MAO-B_a splicing efficiencies with SR proteins (from three independent experiments; $p < 0.05$) quantified by using a scanning imager platform (Fuji FLA-5100 imaging system)

In conclusion, the current study shows that G/A polymorphism in the non-coding sequence of *MAO-B* gene regulates the efficiency of intron 13 removal. This intronic sequence removal might be a rate-limiting factor in the MAO-B mRNA formation leading to the increased protein levels as well as to enzymatic activity in the platelet fraction of patients with Parkinson's disease. Our results suggest that the enhanced MAO-B protein expression levels in platelets might be used as a disease marker.

Part II. Hypoxia-Dependent Alternative pre-mRNA Splicing Regulation in Eukaryotic Cells

In the present study, as a model system, we have used mouse HIF-3α pre-mRNA that is spliced in an oxygen-dependent manner producing HIF-3α (both in normoxia and hypoxia) and IPAS (only in hypoxia) pre-mRNAs (Figure 5A).

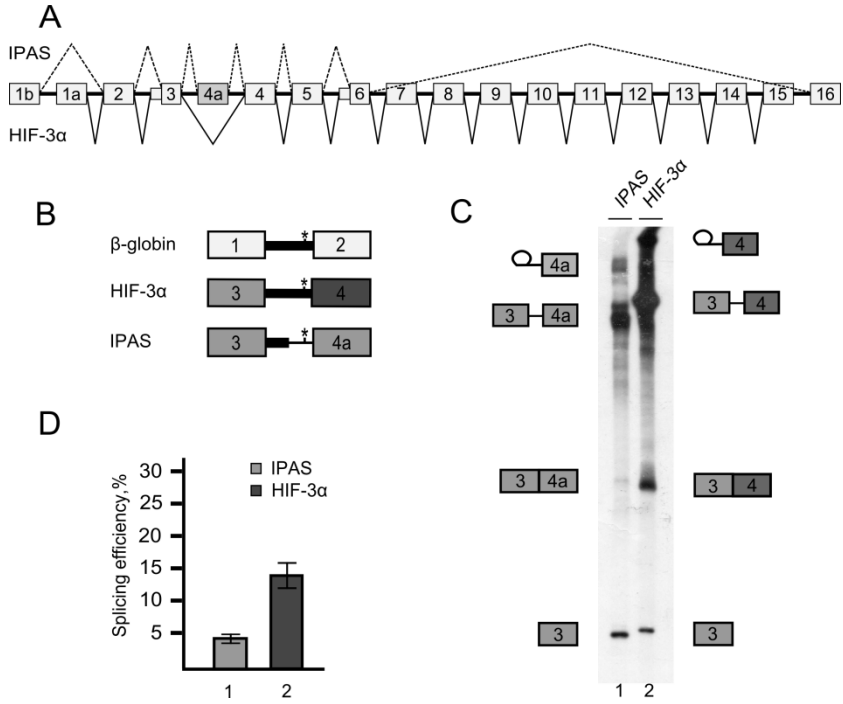

Figure 5. HIF-3α and IPAS constructs are spliced differently in nuclear extracts prepared from HeLa cells cultured under normoxic conditions. (A) Schematic representation of HIF-3α pre-mRNA. (B) Schematic representation of constructs used in *in vitro* splicing assays. The branch point is indicated by an asterisk (*). (C) IPAS and HIF-3α pre-mRNAs splicing in *in vitro* splicing reactions in nuclear extracts prepared from normoxic cells. (D) Calculation of HIF-3α and IPAS splicing efficiencies (from three experiments; $p < 0.05$) by phosphorimaging

We have generated constructs containing the common exon 3 5′ splice site, part of intron 3 and the unique 3′ splice sites of exon 4a (IPAS) and exon 4 (HIF-3α). Rabbit β-globin pre-mRNA served as a positive constitutive splicing control (Figure 5B). All these constructs were tested in *in vitro* splicing assays in nuclear extracts prepared from HeLa cells cultured under normoxic (NE_{nor}) and hypoxic (NE_{hpx}) conditions. The splicing in normoxic nuclear extracts revealed that the

splicing of the IPAS construct was highly inefficient (Figure 5, C and D, lane 1) in contrast to the splicing efficiency of the HIF-3α construct (Figure 5, C and D, lane 2).

Subsequent studies of HIF-3α and IPAS constructs splicing in hypoxic nuclear extracts showed that HIF-3α mRNA formation was barely detectable (Figure 6, A and D, lanes 2–4) in contrast to IPAS spliced product formation (Figure 6, B and E, lanes 2–4). Weakly spliced in hypoxic nuclear extracts, IPAS product formation was observed as early as after 1 hour of reaction (Figure 6, B and E, lane 3). The bands corresponding to the lariat exon 4a intermediate and exon 3 (Figure 6, B and E, lane 3) formation can be seen indicating that, in contrast to the splicing in normoxic extracts, hypoxic nuclear extracts are capable of activating weak 3′ splice site usage. In control experiments, the splicing efficiency of β-globin pre-mRNA decreased about 2-fold in hypoxic nuclear extracts (Figure 6, C and F, lane 2) if compared to normoxic extracts (Figure 6, C and F, lane 1). These results indicate that hypoxic nuclear extracts possess weaker capability of activating a strong β-globin 3' splice site and activate the weak splice site usage within the HIF-3α construct. As nuclear extracts from normoxic or hypoxic cells activate the pre-mRNA splicing differently, we have analyzed the interaction of HIF-3α pre-mRNA with proteins from these extracts in UV crosslinking experiments. The obtained results show that proteins from hypoxic nuclear extracts interact with RNA more efficiently in comparison to proteins from normoxic extracts (Figure 6G). In order to identify the differentially interacting proteins, HIF-3α pre-mRNA was double-labeled ([^{32}P-CTP] and biotin) and crosslinked to proteins from normoxic and hypoxic nuclear extracts by UV radiation. Furthermore, the bound proteins were "fished out" by using streptavidin beads and analyzed by employing Western blot. Western blot analysis of proteins with the anti-SR antibody revealed that SR proteins in nuclear extracts from hypoxic cells interacted more strongly with RNA if compared with those from the normoxic nuclear extract (Figure 6H, lanes 3 and 4).

Next, we have isolated SR proteins from HeLa cells cultured under normoxic (SR_{nor}) and hypoxic conditions (SR_{hpx}) (Wu & Green, 1997) (Figure 10A) and tested their influence on HIF-3α, IPAS and β-globin construct splicing. The addition of SR_{nor} to the splicing reactions in normoxic nuclear extracts activated the β-globin and HIF-3α construct splicing (Figure 7, A and D, lanes 1–3 and 8–11, respectively) and did *not* activate the IPAS construct splicing (Figure 7, A and D, lanes 4–7). The addition of SR_{hpx} to normoxic nuclear extracts activated the splicing of HIF-3α, IPAS and β-globin constructs (Figure 7, B, E and C, F) thus demonstrating that SR_{hpx} possesses a stronger ability to activate splicing as compared to SR_{nor}.

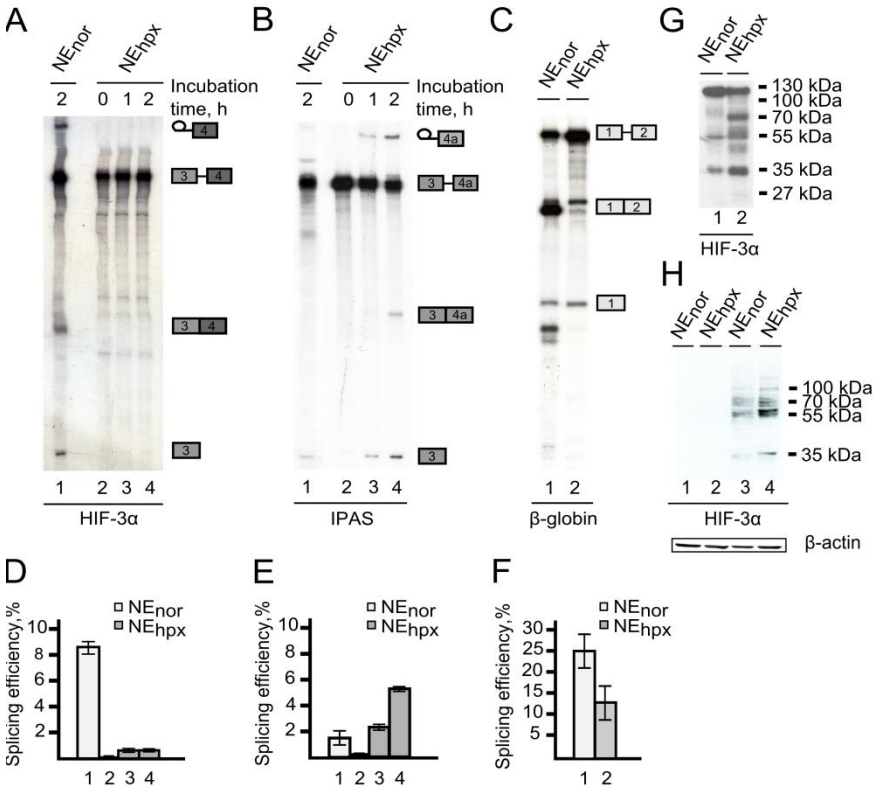

Figure 6. HIF-3α and IPAS constructs in hypoxic extracts are spliced differently. (A) Time course of HIF-3α construct splicing. Lane 1 shows the splicing reaction incubated for 2h in a normoxic nuclear extract, lane 2 represents the reaction incubated for 0h, lane 3 – for 1h, lane 4 – for 2h in hypoxic nuclear extracts. (B) Time course of IPAS (lanes 1–3) construct splicing. Lane 1 shows the splicing reaction incubated for 2h in a normoxic nuclear extract, lane 2 represents the reaction incubated for 0h, lane 3 – for 1h, lane 4 – for 2h in hypoxic nuclear extracts. (C) β-globin construct splicing in normoxic (lane 1) and hypoxic (lane 2) nuclear extracts. (D) HIF-3α, (E) IPAS, (F) β-globin splicing efficiency (from three experiments) quantified by phosphorimaging ($p < 0.05$). (G) Radiogram of UV crosslinked proteins from NE_{nor} and NE_{hpx} with [^{32}P-CTP] labeled HIF-3α pre-mRNA transcript. (H) Western blot analysis of proteins with anti-SR antibody "fished out" from NE_{nor} (lane 3) and NE_{hpx} (lane 4) with streptavidin beads bound to double labeled ([^{32}P-CTP] and biotin) HIF-3α pre-mRNA. "Fishing" controls from NE_{nor} (lane 1) and NE_{hpx} (lane 2) with only [^{32}P-CTP] labeled RNA are shown. β-actin was used as the loading control

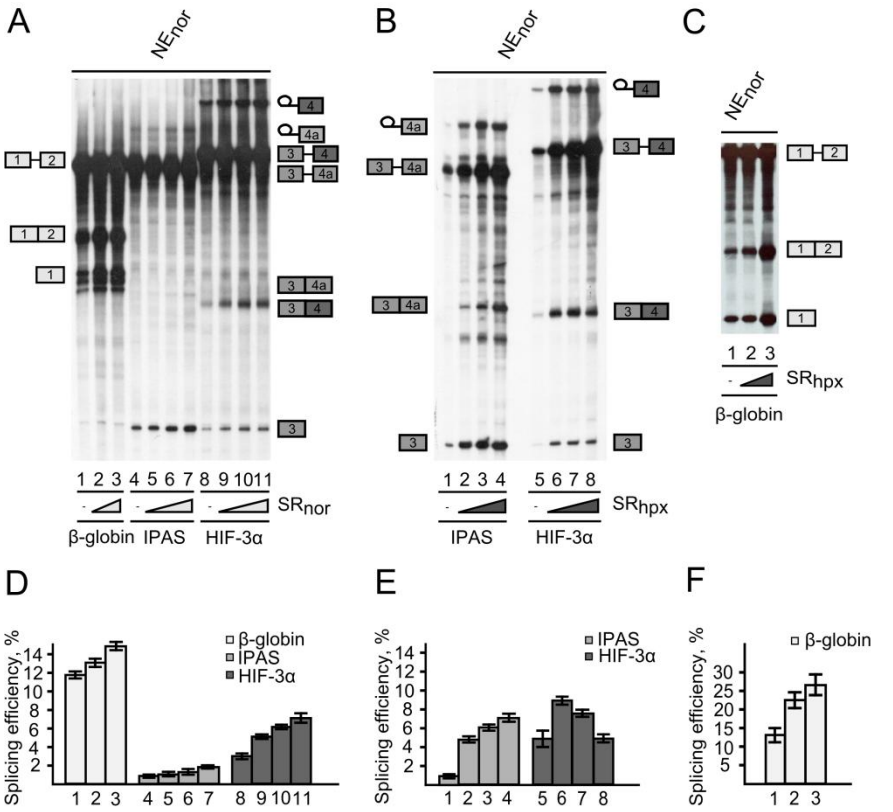

Figure 7. Influence of SR_{nor} and SR_{hpx} on HIF-3α, IPAS and β-globin pre-mRNAs splicing in normoxic HeLa cells nuclear extracts. (A) Splicing of β-globin, IPAS and HIF-3α transcripts (lanes 1,4 and 8, respectively), or with increasing amounts (0.25 – 0.75 μg) of SR_{nor} (lanes 2,3 (β-globin) 5–7 (IPAS) and 9–11 (HIF-3α)). (B). Splicing of IPAS and HIF-3α transcripts (lanes 1 and 5, respectively), or with increasing amounts (0.25 – 0.75 μg) of SR_{hpx} (lanes 2–4 and 6–8, respectively). (C) Splicing of β-globin transcript in NE_{nor} (lane 1) or with increasing amounts (0.25 – 0.5μg) of SR_{hpx} (lanes 2 and 3, respectively). (D) β-globin (lanes 1–3), IPAS (lanes 4–7) and HIF-3α (lanes 8–11) construct splicing efficiencies with SR_{nor}. (E) IPAS (lanes 1–4) and HIF-3α (lanes 5–8) construct splicing efficiencies with SR_{hpx}. (F) β-globin (lanes 1–3) construct splicing efficiencies with SR_{hpx}. Calculation of the spliced product formation (from three experiments; $p < 0.05$) quantified by phosphorimaging

The addition of SR_{nor} to hypoxic nuclear extracts activated the HIF-3α pre-mRNA splicing (Figure 8, A and D, lanes 3 and 4) and slightly activated the IPAS transcript splicing (Figure 8, B and E, lanes 2 and 3). Finally, the addition of SR_{nor} (Figure 8, C and F, lanes 3–5) or SR_{hpx} (Figure 8, C and F, lanes 6–8) proteins to hypoxic nuclear extracts had almost no effect on the β-globin pre-mRNA splicing efficiency. These experiments suggest that SR proteins from hypoxic cells, in comparison with normoxic cells, possess different capacities to activate the splicing of specific splice sites.

As hypoxic cellular effects are reversible, it is possible that the specific SR protein activities in normoxic and hypoxic cells might be reversible. In order to test this hypothesis, SR_{nor} were either added immediately into the splicing reaction (Figure 9, A and B, lanes 1–4) or, before the addition of HIF-3α pre-mRNA, were pre-incubated in normoxic or hypoxic nuclear extracts for 20 min at 30^{o}C temperature (Figure 9, A and B, lanes 5–8). The obtained results showed that the addition of SR_{nor} into the splicing reaction in NE_{nor} and NE_{hpx} activated HIF-3α pre-mRNA splicing (Figure 9, A and B, lanes 1,2 and 3,4). The pre-incubation of SR_{nor} proteins in NE_{nor} resulted in very small differences in terms of their ability to activate HIF-3α construct splicing (Figure 9, A and B, lanes 5 and 6), whereas their ability to activate HIF-3α construct splicing was almost lost upon pre-incubation in NE_{hpx} (Figure 9, A and B, lanes 7 and 8). It means that the pre-incubation of SR_{nor} proteins in hypoxic extracts reduced their ability to activate splicing.

It has been demonstrated that the RS domain of SR proteins is phosphorylated (Howard & Sanford, 2015). As we have found that the steady-state concentrations of SR proteins do not change in normoxic and hypoxic HeLa cells (Figure 10A), we have used Western blot with mAb104 antibody assays while striving to assess the modification status of SR proteins. The results show that SR proteins isolated from hypoxic cells were less efficiently recognized by mAb104 (Figure 10B). This data suggests that the modification status of the SR protein RS domain in hypoxic cells might be different if compared to normoxic cells. In order to define the differences in SR proteins modification, we have used two-dimensional (2D) gel analysis. The Western blot of 2D gel with anti-SR antibody analysis has revealed that a large fraction of SR_{hpx} was significantly shifted towards the acidic side of the gel (Figure 10C) thus indicating that hypoxic SR proteins are hyper-phosphorylated if compared to the SR proteins from normoxic extracts.

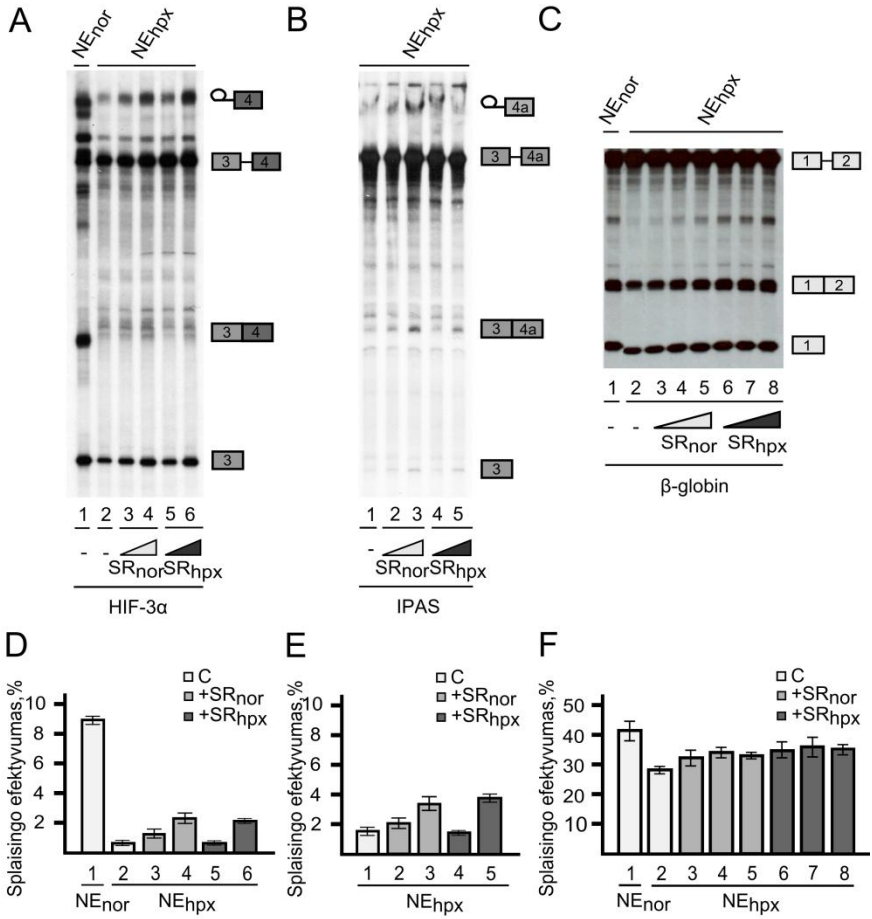

Figure 8. Influence of SR_{nor} and SR_{hpx} on HIF-3α, IPAS and β-globin pre-mRNAs splicing in hypoxic HeLa cells nuclear extracts. (A) Splicing of HIF-3α transcript in NE_{nor} (lane 1) and NE_{hpx} (lane 2), or with increasing amounts (0.25 – 0.5 μg) of SR_{nor} (lanes 3,4) or SR_{hpx} (lanes 5,6). (B) Splicing of IPAS transcript in NE_{hpx} (lane 1) or with increasing amounts (0.25 – 0.5 μg) of SR_{nor} (lanes 2,3), or SR_{hpx} (lanes 4,5). (C) Splicing of β-globin transcript in NE_{nor} (lane 1) and NE_{hpx} (lane 2), or with increasing amounts (0.25 – 0.75μg) of SR_{nor} (lanes 3–5) or SR_{hpx} (lanes 6–8). (D) HIF-3α, (E) IPAS, (F) β-globin construct splicing efficiencies with SR_{nor} and SR_{hpx} proteins (from three experiments; $p < 0.05$) quantified by phosphorimaging. C highlights the splicing efficiency in nuclear extracts without additional SR proteins

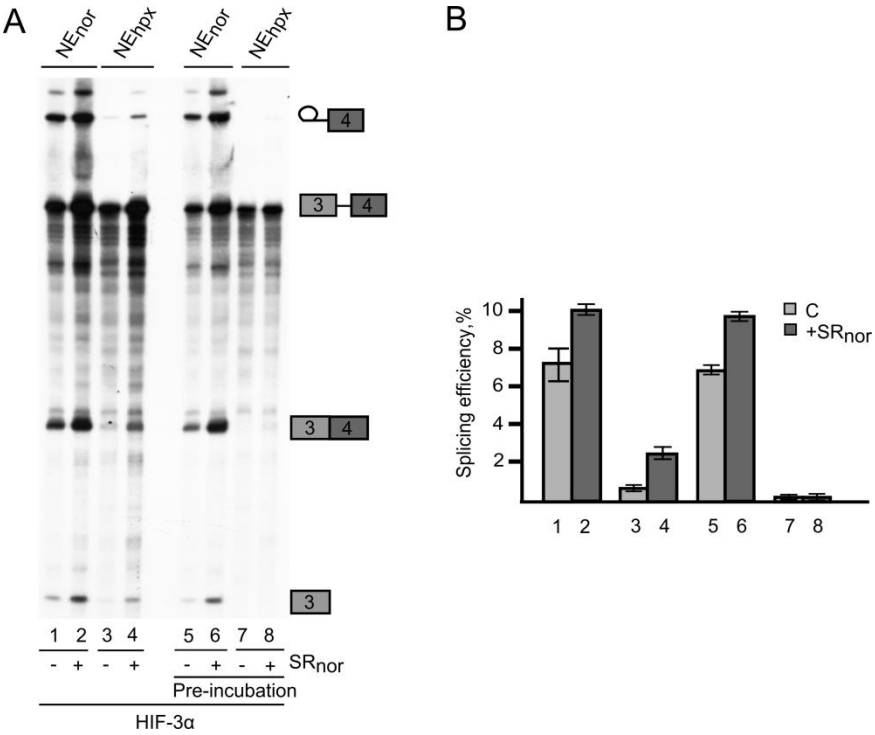

Figure 9. Preincubation of SR_{nor} in hypoxic nuclear extract results in the loss of ability to activate HIF-3α pre-mRNA splicing. (A) HIF-3α transcript splicing in NE_{nor} (lanes 1, 2, 5, 6) or NE_{hpx} (lanes 3, 4, 7, 8). 0,5 μg of SR_{nor} was added into the splicing reactions (lanes 2, 4, 6, 8). Before the addition of RNA into the splicing reaction, SR proteins were pre-incubated either in NE_{nor} (lane 6) or in NE_{hpx} (lane 8) for 20 min at 30^{o}C. (B) Average of HIF-3α construct splicing efficiencies (from three experiments; $p < 0.05$) quantified by phosphorimaging

It is known that serine residues within the RS domain of SR proteins are phosphorylated by specific SR protein kinases (CLK1 and SRPK1, SRPK2) (Ghosh & Adams, 2011). We have compared SR protein kinase expression levels in HeLa cells cultivated under normoxic or hypoxic conditions. We have consequently found out that the expression of CLK1, SRPK1 and SRPK2 was elevated at the mRNA and protein levels in hypoxic cells compared with normoxic cells (Figures 10D and E).

The observation that the expression of CLK1, SRPK1 and SRPK2 kinases in hypoxic cells is increased raised the question of hypoxia-inducible factor HIF-1 involvement in the regulation of SR protein kinases expression. By using the bioinformatics analysis of the *CLK1*, *SRPK1* and *SRPK2* promoter regions, we

have found out that there are five potential HIF binding sites (5'-d(RCGTG)-3', R = A or G) within the *CLK1* gene promoter region. Meanwhile, we have not detected any HIF binding sites within the *SRPK1* and *SRPK2* genes promoter regions. In order to investigate if the enhanced expression of CLK1 kinase in hypoxic cells is regulated by HIF-1, we have used anti-HIF-1α antibodies so that to perform chromatin immunoprecipitation (ChIP) experiments. Immunoprecipitated DNA was subjected to real-time PCR with primers that amplify a promoter fragment spanning potential HIF binding sites. These experiments showed a 3-fold increase in amplification of the *CLK1* promoter region in extracts from hypoxic cells compared to normoxic cells thus demonstrating that HIF-1 is recruited to the *CLK1* promoter region in a hypoxia-dependent manner (Figure 10F).

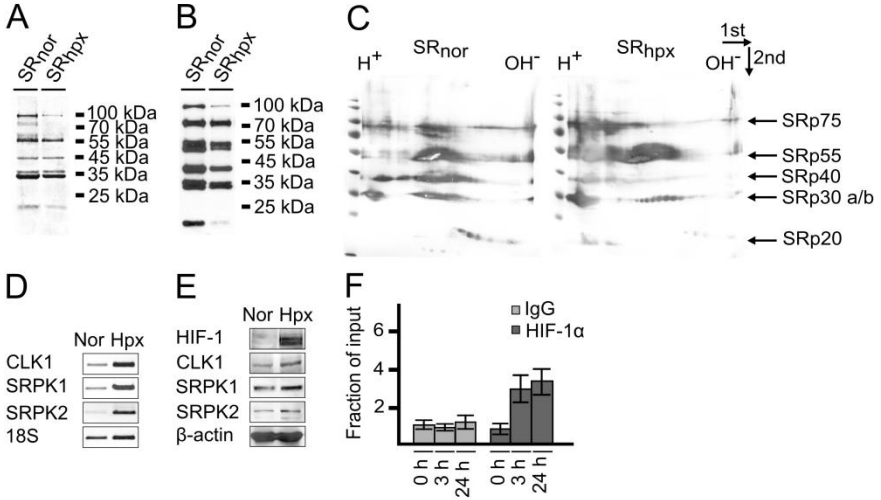

Figure 10. Hypoxia induces SR protein hyperphoshorylation by hypoxia-dependent increase in CLK1 kinase cellular levels. (A) SDS-PAGE of SR_{nor} and SR_{hpx} proteins. (B) Western blot analysis of SR_{nor} and SR_{hpx} proteins with mAb104 antibody. (C) Western blot of two-dimensional (2D) gel electrophoresis of SR_{nor} and SR_{hpx} proteins with anti-SR antibody. (D) CLK1, SRPK1 and SRPK2 expression at mRNA level in hypoxic and normoxic HeLa cells. (E) CLK1, SRPK1 and SRPK2 protein expression in hypoxic and normoxic HeLa cells. (F) ChIP to monitor recruitment of HIF-1α to the CLK1 promoter under hypoxic conditions (0–24 h). Antibodies against IgG and HIF-1α were used. Potential HIF-1α binding sites within the CLK1 promoter were amplified by using real-time PCR as described in the *materials and methods* part. Each bar represents the mean ±SD of three to five independent experiments; $p < 0.05$ comparing 21% *versus* 1% O_2

In order to verify the influence of the CLK1 expression in hypoxia-dependent splicing, as a model system, we have used endogenous pre-mRNAs of well-characterized hypoxia target genes, such as carbonic anhydrase IX (CAIX) and cysteine-rich angiogenic inducer 61 (Cyr61). CAIX is a hypoxia-induced, tumor-associated carbonic anhydrase with functional involvement in pH control and cell adhesion. It has been reported that two alternatively spliced mRNAs isoforms (AS CAIX and FL CAIX) are produced from CAIX pre-mRNA. The human alternatively spliced AS CAIX isoform does not contain exons 8–9 and is produced in very low amounts. FL CAIX mRNA production is hypoxia-inducible (Barathova *et al*, 2008). Cyr61 protein is involved in cell adhesion, migration, proliferation and apoptosis. Cyr61 protein is down-regulated in prostate cancer and leiomyoma (Perbal, 2009). This fact suggests that, under certain circumstances, Cyr61 might behave as a tumor suppressor. Two alternatively spliced isoforms are generated from Cyr61 pre-mRNAs. One isoform contains the correctly spliced mRNA (joint all 5 exons or intron skipping (IS) isoform), or another isoform between exons 3 and 4 contains intron 3 retention (intron-retaining (IR) isoform) showing that RNA alternative splicing might play an important role in the expression regulation of this protein. The retention of intron 3 creates two-stop codons within the intronic sequence. It is not known whether a truncated protein is expressed from the Cyr61 IR mRNA (Holbourn *et al*, 2009).

At first, we monitored the effects of siRNA-mediated down-regulation of CLK1 expression on CAIX and Cyr61 pre-mRNA splicing (Figure 11A). A slight reduction of AS CAIX isoform was observed in normoxic HeLa cells treated with CLK1 siRNA (Figure 11A, lane 2). The expressions of both CAIX mRNA isoforms are enhanced in hypoxic cells (Figure 11A, lanes 3 and 4). The reduction of CLK1 levels by specific siRNA treatment almost does not affect AS CAIX mRNA isoform formation and reduces FL CAIX isoform formation in cells (Figure 11A, lane 4). In addition, the reduction of CLK1 expression decreases not only FL CAIX mRNA, but also the CAIX cellular protein level (Figure 11C, lane 4) thus indicating that, for the enhanced expression of CAIX in hypoxic cells, the increased CLK1 expression is also needed.

In the Cyr61 pre-mRNA alternative splicing case in hypoxic cells, the formation of Cyr61 IR mRNA isoform is promoted (Figure 11, A and D, lanes 1 and 3). The obtained results demonstrate that the reduction of CLK1 cellular expression levels changes the Cyr61 mRNA isoform expression pattern from IS to IR (Figure 11, A and D).

In order to further investigate the effects of SR protein hyper-phosphorylation on hypoxia-dependent splicing, cells cultivated either under normoxic or hypoxic conditions were treated with CLK1 kinase inhibitor TG003 (Muraki *et al*, 2004). The inhibition of kinase activity revealed that CAIX (Figure 11B) and Cyr61 (Figure 11, B and E, lanes 1 and 3) mRNA isoform

formation is similar to the one observed in the cells treated with CLK1 siRNA. This data suggests that the CLK1 cellular protein level/activity is an important factor for the hypoxia-dependent splicing regulation.

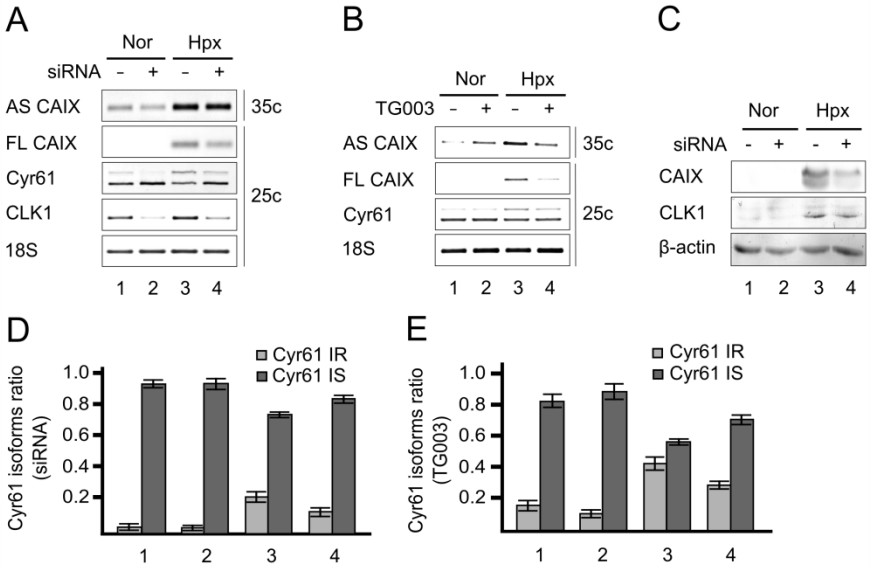

Figure 11. Reduction of CLK1 protein levels or kinase activity changes hypoxia-dependent CAIX and Cyr61 alternative pre-mRNA splicing. (A) siRNA-mediated reduction of CLK1 mRNA expression correlated with the reduced formation of FL CAIX mRNA and changed Cyr61 pre-mRNA alternative splicing in hypoxic cells. (-) Normoxic (lane 1) and hypoxic (lane 3) HeLa cells treated with control siRNA. (+) Normoxic (lane 2) and hypoxic (lane 4) HeLa cells treated with CLK1 specific siRNA. (B) Cell treatment with CLK1 kinase inhibitor TG003 correlated with the reduced formation of FL CAIX mRNA and changed Cyr61 pre-mRNA alternative splicing in hypoxic cells. (-) Normoxic (lane 1) and hypoxic (lane 3) HeLa cells untreated with TG003. (+) Normoxic (lane 2) and hypoxic (lane 4) HeLa cells treated with TG003. (C) Normoxic or hypoxic cell treatment with CLK1-specific siRNA reduces CLK1 (lanes 2 and 3) and FL CAIX cellular protein levels. (-) Normoxic (lane 1) and hypoxic (lane 3) HeLa cells treated with control siRNA. (+) Normoxic (lane 2) and hypoxic (lane 4) HeLa cells treated with CLK1-specific siRNA. (D) Quantitation of Cyr61 mRNA IR and IS isoform relative ratio from normoxic (lanes 1 and 2) or hypoxic (lanes 3 and 4) cells treated with non-specific siRNA (lanes 1 and 3) or with CLK1-specific siRNA (lanes 2 and 4). (E) Quantitation of Cyr61 mRNA IR and IS isoform relative ratio from normoxic (lanes 1 and 2) or hypoxic (lanes 3 and 4) cells untreated with CLK1 inhibitor TG003 (lanes 1 and 3) or treated with TG003 (lanes 2 and 4). 35c and 25c – 35 or 25 PCR cycles were performed (Cyr61 isoforms ratio were calculated from three experiments; $p < 0.05$)

In summary, the current study shows that SR proteins are one of hypoxia-dependent pre-mRNA splicing regulators. Hypoxia changes the activity of SR proteins thus increasing their phosphorylation levels following the up-regulation of the expression of SR protein kinases.

This leads to a different mRNA isoform formation in order to adapt the cell to the reduced oxygen tension.

General Conclusions

1. The enhanced MAO-B protein levels in the platelets from patients with Parkinson's disease compared to healthy individuals might be used as a disease marker.
2. The "A" allele in a non-coding sequence increases the efficiency of the intronic sequence removal.
3. Single nucleotide polymorphism in the intronic sequence is sufficient to influence the pre-mRNA splicing efficiency.
4. G/A polymorphism in *MAO-B* gene intron 13 affects the interaction of SR proteins with pre-mRNA: A nucleotide creates SR protein binding site.
5. SR proteins isolated from HeLa cells cultured under hypoxia interact with RNA more efficiently if compared to SR proteins isolated from HeLa cells cultured under normoxic conditions.
6. SR proteins isolated from hypoxic HeLa cells are more phosphorylated than SR proteins isolated from normoxic HeLa cells.
7. The enhanced SR protein phosphorylation level in hypoxic HeLa cells is caused by an increased expression of specific SR protein kinases (CLK1 ir SRPK1, SRPK2).
8. An increase in the expression of CLK1 kinase is regulated by hypoxia inducible transcription factor 1 (HIF-1).
9. The reduction of CLK1 cellular expression levels alters the hypoxia-dependent CAIX and Cyr61 alternative pre-mRNA splicing: FL CAIX mRNA and protein formation is reduced, whereas Cyr61 IS mRNA isoform formation is promoted.

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List of Publications on the Theme of the Dissertation

Articles in the Journals Included in the List of the Institute for Scientific information ISI Web of Science

1. **Jakubauskienė, E.**, Janavičiūtė, V., Pečiulienė, I., Söderkvist, P., Kanopka, A. (2012). G/A Polymorphism in Intronic Sequence Affects the Processing of MAO-B Gene in Patients with Parkinson's Disease. In: *FEBS Letters*. 2012, 586(20), p. 3698–3704 (ISI Web of Science).
2. **Jakubauskienė, E.**, Vilys, L., Makino, Y., Poellinger, L., Kanopka, A. (2015). Cellular Hypoxia Changes Alternative pre-mRNA Splicing by Regulating SR Protein Phosphorylation Levels. In: *The Journal of Biological Chemistry*. 2015, 290(29), p. 18079–18089 (ISI Web of Science).

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1. **Jakubauskienė, E.**, Kanopka, A. (2012). Polymorphism in Monoamine Oxidase B Gene in Related with Parkinson's Disease Influence of pre-mRNA Splicing. In: *Mokslas – sveikatai*, LSMU, Kaunas, Lithuania.
2. **Jakubauskienė, E.**, Vilys, L., Makino, Y., Poellinger, L., Kanopka, A. (2013). SR Proteins Regulate from Cellular Environment Dependent Splicing. In: *Dealing with Hypoxia: Regulatory Aspects in Cells, Tissues and Organisms*. Oulu, Finland.
3. **Jakubauskienė, E.**, Vilys, L., Makino, Y., Poellinger, L., Kanopka, A. (2013). SR Proteins Regulate from Cellular Environment Dependent Splicing. In: *The 18th Annual Meeting of the RNA Society*. Davos, Switzerland.
4. **Jakubauskienė, E.**, Butkytė, S., Ščerbakovaitė, A., Vilkaitis, G., Kanopka, A. (2014). The Expression of Splicing Factors in Digestive System Tumours and Cell Lines. In: *FEBS-EMBO*. Paris, France.
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REZIUMĖ

Darbo aktualumas

Pastaraisiais dešimtmečiais vis didėja sergamumas įvairiomis ligomis, todėl kyla išsamios informacijos apie ląstelėje vykstančių biologinių procesų pokyčius poreikis. Siekdami išsiaiškinti ir pašalinti ligų vystymosi žmogaus organizme priežastis, daug pasaulio mokslininkų atlieka molekulinės ligų patogenezės tyrimus. Viena iš įvairių ligų atsiradimo priežasčių yra pakitęs pre-iRNR splaisingas. Ląstelės branduolyje vykstančio pre-iRNR splaisingo metu nekoduojančios pre-iRNR sekos yra pašalinamos, o baltymą koduojančios sekos sujungiamos. Šio proceso netikslumai nulemia pakitusių iRNR izoformų susidarymą ir genų raiškos pokyčius, siejamus su neurodegeneracinių, onkologinių, plaučių, širdies ir kraujagyslių ligų atsiradimu ir progresavimu. Todėl pre-iRNR splaisingo pokyčių ląstelėje tyrimai išlieka aktualūs, o atlikti darbai papildo žinias apie daugybės ligų vystymosi priežastis.

Šiame moksliniame darbe aprašomi du tyrimo objektai: *monoaminooksidazės B (MAO-B)* geno polimorfizmo, esančio nekoduojančioje sekoje, įtaka iRNR susidarymui ir nuo sumažėjusio deguonies kiekio aplinkoje priklausomų genų pre-iRNR splaisingas.

Lėtinė neurodegeneracinė Parkinsono liga (PL), atsirandanti 50–70 metų amžiuje, pasireiškia tam tikros smegenų srities (*substantia nigra*), kontroliuojančios valingus judesius, pakitimais. Viena iš šios ligos priežasčių yra *MAO-B* geno nekoduojančioje sekoje aptinkamas vieno nukleotido polimorfizmas – G nukleotido pakeitimas į A nukleotidą. Rengiant šį darbą atlikti tyrimai parodė, kad šis polimorfizmas lemia nekoduojančios sekos pašalinimo efektyvumą, nuo kurio priklauso susidariusios MAO-B iRNR kiekis ir baltymo raiška. Nustačius, kad sveikų ir Parkinsono liga sergančių žmonių kraujyje skiriasi MAO-B baltymo raiška, tokio pobūdžio tyrimai gali būti panaudojami diagnozuojant Parkinsono ligą. Atliktas darbas suteikia vertingos informacijos apie *MAO-B* geno nekoduojančioje sekoje esančio G/A polimorfizmo įtaką pre-iRNR splaisingui, kartu ir Parkinsono ligos patogenezei.

Daugybės ligų, pvz., infarkto, išeminės širdies ligos, periferinių ir koronarinių arterijų ligų, širdies nepakankamumo, anemijos, plaučių arterijos hipertenzijos, obstrukcinės miego apnėjos, eritrocitozės, onkologinių ligų, atsiradimas ir vystymasis susijęs su sumažėjusiu deguonies kiekiu ląstelėje. Deguonies trūkumas (hipoksija) stabilizuoja hipoksijos indukuojamus veiksnius (HIF), aktyvinančius genų, reikalingų ląstelei išgyventi hipoksinėmis sąlygomis, transkripciją. Ląstelei prisitaikant prie aplinkos pokyčių didelę reikšmę turi alternatyvusis pre-iRNR splaisingas, kurio metu nuo tos pačios pre-iRNR susidaro skirtingus baltymus koduojančios iRNR izoformos. Šių izoformų susidarymui įtakos turi pakitęs splaisingo veiksnių aktyvumas. Nustatyta, kad pakitusi ląstelės aplinka lemia pre-iRNR splaisingo pokyčius, tačiau šį procesą

reguliuojantys veiksniai iki šiol nėra nustatyti. Todėl nuo deguonies kiekio aplinkoje priklausomo alternatyviojo pre-iRNR splaisingo ir jį reguliuojančių veiksnių tyrimai yra aktualūs siekiant išsiaiškinti šio proceso pokyčių priežastis ir rasti būdų slopinti hipoksinėse ląstelėse pakitusių iRNR susidarymą, kartu lėtinant ligų vystymąsi.

Darbo tikslai ir uždaviniai

Šio darbo **tikslai**:

1. Ištirti *MAO-B* geno 13-o introno sekoje aptinkamo G/A polimorfizmo įtaką pre-iRNR splaisingui, nustatyti splaisingo reguliacijos procese dalyvaujančius veiksnius ir MAO-B baltymo raiškos lygio pokyčius Parkinsono liga sergančių ir sveikų žmonių kraujo trombocitų frakcijoje.
2. Nustatyti veiksnius, reguliuojančius nuo deguonies koncentracijos aplinkoje priklausomą alternatyvųjį pre-iRNR splaisingą, ir rasti būdų kontroliuoti nuo hipoksijos priklausomų alternatyvių pre-iRNR splaisingo taikinių pasirinkimą.

Pirmajam tikslui pasiekti buvo iškelti šie **uždaviniai:**

1. Nustatyti, ar MAO-B baltymo raiškos lygio pokyčiai Parkinsono liga sergančių ir sveikų asmenų kraujo trombocitų frakcijoje gali būti naudojami Parkinsono ligos diagnostikoje.
2. Išsiaiškinti G/A polimorfizmo, aptinkamo *MAO-B* geno 13-o introno sekoje, įtaką pre-iRNR splaisingui.
3. Nustatyti veiksnius, darančius įtaką MAO-B pre-iRNR splaisingui.

Antrajam tikslui pasiekti buvo suformuluoti šie **uždaviniai:**

1. Nustatyti veiksnius, darančius įtaką nuo deguonies kiekio aplinkoje priklausomam splaisingui, kaip modelinę sistemą naudojant pelės HIF-3α pre-iRNR.
2. Išsiaiškinti, kas lemia veiksnių, reguliuojančių nuo hipoksijos priklausomą pre-iRNR splaisingą, pokyčius.
3. Patvirtinti gautus *in vitro* splaisingo reguliavimo rezultatus ląstelėse, naudojant endogenines pre-iRNR, kurių alternatyvusis pre-iRNR splaisingas priklauso nuo hipoksijos.

Darbo mokslinis naujumas ir praktinė vertė

Disertacinis darbas sudarytas iš dviejų dalių, kuriose tyrinėjamos konstitutyviojo ir alternatyviojo pre-iRNR splaisingo pokyčių priežastys ir jų sąsajos su žmogaus ligomis.

Pirmoje dalyje atlikti išsamūs vieno nukleotido polimorfizmo, esančio *MAO-B* geno nekoduojančioje sekoje, įtakos pre-iRNR splaisingui tyrimai. Duomenų apie tokius tyrimus literatūroje skelbta nebuvo. Pirmą kartą eksperimentais parodyta, kad G/A polimorfizmas 13-o introno sekoje nulemia nekoduojančios sekos pašalinimo iš MAO-B pre-iRNR efektyvumą ir kartu

reguliuoja MAO-B iRNR susidarymo greitį. Pademonstruota, kad itin svarbias funkcijas pre-iRNR splaisingo procese atliekanti SR baltymų šeima skirtingai sąveikauja su laukinio tipo („G“ alelis) ir mutuota („A“ alelis) *MAO-B* geno 13-o introno seka. Ištirta, kad SR šeimos baltymai, stipriau sąveikaujantys su mutuota *MAO-B* geno 13-o introno seka, lemia padidėjusį splaisosomos komplekso susidarymo greitį, o tai daro įtaką nekoduojančios sekos pašalinimui iš pre-iRNR. Gauti nauji duomenys leidžia paaiškinti padidėjusios MAO-B raiškos Parkinsono ligos atveju priežastis, o šio baltymo raiškos pokyčiai gali būti panaudojami kaip Parkinsono ligos žymuo.

Antroje mokslinio darbo dalyje išsamiai apibūdinti nuo hipoksijos priklausomo alternatyviojo pre-iRNR splaisingo ir šį procesą reguliuojančių veiksnių tyrimai. Splaisingo tyrimuose *in vitro* kaip modelinė sistema naudota pelės HIF-3α pre-iRNR. Pademonstruota, kad hipoksinėse (1 % O_2) ląstelėse SR šeimos baltymų aktyvumai skiriasi nuo šių baltymų aktyvumų normaliomis deguonies (21 % O_2) sąlygomis kultivuotose ląstelėse. Atlikus nuodugnius SR šeimos baltymų, išskirtų iš normaliomis deguonies ir hipoksinėmis sąlygomis augintų ląstelių, tyrimus, buvo nustatyta, kad aktyvumų skirtumus lemia skirtingi šių baltymų modifikacijos lygiai. Pirmą kartą buvo parodyta, kad padidėjusį SR baltymų fosforilinimą hipoksinėse ląstelėse lemia padidėjusi specifinių SR šeimos baltymų kinazių (SRPK1, SRPK2 ir CLK1) raiška. Padidėjusią kinazės CLK1 raišką lemia hipoksijos indukuojamas transkripcijos veiksnys HIF-1, aktyvuojantis šio geno transkripciją hipoksinėse ląstelėse. *In vitro* sistemoje gauti rezultatai patvirtinti eksperimentais ląstelėse, naudojant endogenines pre-iRNR, kurių alternatyvusis splaisingas reguliuojamas priklausomai nuo deguonies kiekio aplinkoje. Rengiant šį darbą atlikti tyrimai yra itin vertingi siekiant išsiaiškinti hipoksinėse ląstelėse vykstančių procesų priežastis, o gauti rezultatai suteikia galimybę reguliuoti nuo hipoksijos priklausomą pre-iRNR splaisingą. Remiantis šiais naujais duomenimis, galima slopinti hipoksinėse ląstelėse pakitusių iRNR susidarymą, užkertant kelią ligų vystymuisi žmogaus organizme.

Pirmosios darbo dalies ginamieji teiginiai

1. MAO-B baltymo raiškos lygio pokytis Parkinsono liga sergančių ir sveikų asmenų kraujo trombocitų frakcijoje gali būti naudojamas kaip naujas žymuo diagnozuojant Parkinsono ligą.
2. *MAO-B* geno 13-o introno sekoje esantis G/A polimorfizmas daro įtaką pre-iRNR splaisingui.
3. Vieni iš MAO-B raiškos reguliatorių yra splaisingo veiksnių grupė – SR šeimos baltymai.

Antrosios darbo dalies ginamieji teiginiai

1. Deguonies kiekio aplinkoje pokyčiai lemia pakitusį vienų iš splaisingo veiksnių – SR šeimos baltymų aktyvumą.
2. Skirtingą SR šeimos baltymų aktyvumą hipoksinėse ląstelėse lemia pakitusi šių baltymų RS motyvų modifikacija.
3. Skirtingus SR baltymų modifikacijos lygius lemia specifinių SR baltymų kinazių raiška.
4. CLK1 raiškos sumažinimas keičia nuo hipoksijos priklausomą alternatyvųjį pre-iRNR splaisingą ląstelėse.

Bendrosios išvados

1. Parkinsono ligos atveju padidėjęs MAO-B baltymo raiškos lygis (palyginti su raiška sveikame žmoguje) yra tinkamas naudoti diagnozuojant Parkinsono ligą žmogaus organizme.
2. „A“ alelį turinti nekoduojanti seka padidina introninės sekos pašalinamo efektyvumą.
3. G/A nukleotido polimorfizmas nėra specifinis tik *MAO-B* geno 13-o introno pašalinimo efektyvumui.
4. *MAO-B* geno 13-o introno sekoje esantis G/A polimorfizmas lemia SR šeimos baltymų sąveikos su pre-iRNR pokyčius: A nukleotidą turinti introninė seka pagreitina splaisosomos komplekso susidarymą, o tai padidina nekoduojančios sekos pašalinimo efektyvumą.
5. SR šeimos baltymai, išgryninti iš hipoksinėmis sąlygomis augintų HeLa ląstelių, pasižymi didesniu gebėjimu aktyvinti pre-iRNR splaisingą nei SR šeimos baltymai, išskirti iš normaliomis deguonies sąlygomis kultivuotų HeLa ląstelių.
6. SR šeimos baltymų, išskirtų iš hipoksinėmis sąlygomis augintų ląstelių, fosforilinimo lygis yra didesnis už SR šeimos baltymų, išskirtų iš normaliomis deguonies sąlygomis augintų ląstelių, fosforilinimo lygį.
7. Padidėjusį SR šeimos baltymų fosforilinimo lygį hipoksinėse ląstelėse lemia padidėjusi specifinių SR baltymų kinazių (CLK1 ir SRPK1, SRPK2) iRNR ir baltymo raiška.
8. CLK1 kinazės padidėjusią iRNR ir baltymo raišką hipoksinėse ląstelėse lemia hipoksijos indukuojamas transkripcijos veiksnys HIF-1.
9. CLK1 kinazės raiškos sumažinimas pakeičia karboanhidrazės IX ir daug cisteino turinčio angiogenezės aktyviklio 61 alternatyvųjį pre-iRNR splaisingą ląstelėje: slopinamas hipoksinėmis sąlygomis susidariusių iRNR izoformų formavimasis ir skatinamas normaliomis deguonies sąlygomis besiformuojančių iRNR izoformų susidarymas.

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