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Transcriptional and post-transcriptional control of eEF1A2 expression during myoblast diffrerentiation

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During postnatal development, the switch of the expression from isoform A1 to the isoform A2 of eukaryotic translation elongation factor (eEF1A) is observed in neuronal and muscle tissues. The switch of the expression is a vital fundamental process, as mutant mice, with the partial EEF1A2 deletion dies on the 28th day after birth. Mechanism of the inhibition of A1 and stimulation of A2 expression during the first days of postnatal development is unknown. The existence of potential miRNA binding sites in the 3'UTR of mRNAs encoding the isoforms assumes a post-transcriptional control of abovementioned phenomenon. ***Aim****. To check the possibility of post-transcriptional regulation of the isoforms A1 and A2 expression during differentiation of the human immorta- lized myoblasts cell line LHCN.* ***Methods****. The level of gene expression was quantified by qPCR, the existence of post-transcriptional regulation was demonstrated with Dual-Luciferase® Reporter Assay.* ***Results****. Using immortalized human myoblasts cell line LHCN, the induction of isoform A2 of eEF1 during differentiation of myoblasts was shown. The existence of transcriptional and post-transcriptional control of the abovementioned process was confirmed. Downregulation of mir-661 and mir-744 that have binding sites in the 3' UTR of EEF1A2 mRNA, during differentiation suggests a potential role of microRNAs in the eEF1A2 induction during myoblast differentiation.* ***Conclusions****. Induction of A2 isoform of eEF1 during differentiation of myoblasts occurs on transcriptional and post-transcriptional level.*

Keywords: eEF1A1, eEF1A2, immortalized human myoblasts LHCN, differentiation, microRNA.

Introduction. Eukaryotic translation elongation factor (eEF1A) is one of crucial elements of protein synthesis [1]. The main eEF1A function is the delivery of aminoacyl tRNAs to A-site of ribosome [2]. The isoform A1, eEF1A1, is more widespread and expressed in all tissues during embryogenesis. However, during the postnatal period of development some tissues showed the decrease and standstill of the eEF1A1 expression accompanied by the induction of the expression of the A2 isoform, eEF1A2 [3, 4]. Such changes are specific exclusively for terminally differentiated cells, particularly neurons, cardiomyocytes, and myocytes [5, 6]. As of now, the question about the mechanism and physiological significance of the switch of the expression of isoforms remains unsolved. The absence of A2 isoform as a result of the partial deletion of EEF1A2 gene in *wst/wst* mutant mice, was lethal. These mice showed gradual downregulation in A1 expression with no replacement by A2. The phenotype of such mice is described by the loss of body weight, tremor, pathologies of muscular and nervous systems. On day 28 after the birth, these allele homozygotes died [7].

These isoforms are 98 % homologous, yet, regardless of high homology and probably similar translation efficiency, these proteins differ significantly in their non-canonical functions [8, 9]. eEF1A1 shows pro-apoptotic features, whereas eEF1A2 is antiapoptotic [10]. There are data confirming proto-oncogenic nature of eEF1A2 [11]. A2 isoform was shown to appear in

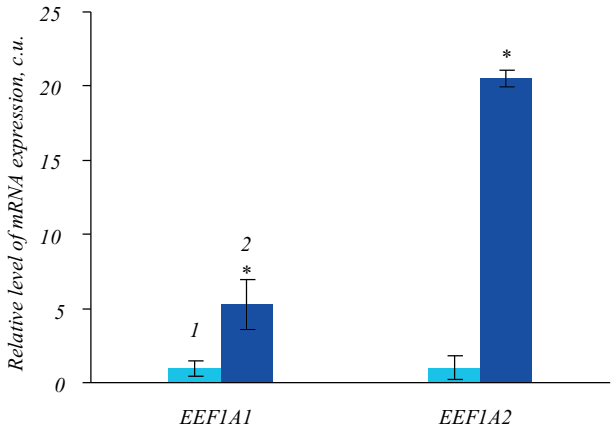

Fig. 1 The switch in the expression of isoforms of elongation translation factor eEF1 during the differentiation of myoblasts. The quantitative PCR-analysis of mRNA of EEF1A1/A2 in proliferating myoblasts (*1*) and differentiated myocytes (*2*); *$p < 0.05$; *t*-Student's test

cancer tissues of mammary gland, lungs, and ovaries [12].

Regardless of high protein homology, mRNA encoding the A1 and A2 isoforms differ in 3' and 5' untranslated regions (UTR) considerably, which suggests probability of different control of post transcriptional expression of isoforms. MicroRNA is known to be one of principal participants of the post-transcriptional control system [13]. The existence of sites specific for different microRNAs in UTRs of mRNA coding for A1 or A2 suggests their participation in the post-transcriptional control of the expression switch from the A1 to A2 isoform. This paper presents experimentally confirmed hypothesis about possibility of post-transcriptional control in the expression switch between the eEF1A isoforms during differentiation of myoblasts. The involvement of microRNA in this process is shown.

Materials and Methods. *Cell culture*. Cultivation and differentiation of immortalized human myoblasts LHCN were performed as described in [14].

cDNA synthesis, qPCR. Total RNA was extracted from (1-2)·10^6 cells, using TRI-reagent (Sigma Chem. Co, USA). cDNA was synthesized by reverse transcriptase RevertAid (Fermentas, Lithuania) and oligod T-primer following the manufacturer's recommendations. Quantitative PCR was performed as described in [15]. Relative quantity of mRNA eEF1A1/A2 was normalized to beta-actin mRNA. Quantitative PCR-analysis of microRNA was performed using TaqMan® MicroRNA Assays kit (Applied Biosystems, USA), following the manufacturer's recommendations.

Reporter gene assay. Proliferating or differentiated LHCN cells were transfected with 20 ng of plasmid, containing the open reading frame of luciferase and 3'-UTR of EEF1A1/A2 mRNA using Lipofectamine 2000 (Invitrogene, USA), following the manufacturer's instructions. 24 hours later the luciferase level was measured using Dual-Luciferase® Reporter Assay System as described by the manufacturer.

Results and Discussion. The immortalized human myoblasts were obtained by the transformation with telomerase and cyclin-dependent kinase 4 [14]. The obtained cell line was capable of proliferation as non-differentiated myoblasts as well as differentiating myoblasts with subsequent formation of myocytes. The main criterion for the use of LHCN cells for studying the regulation of A1 and A2 expression was switchable expression of the isoforms after the differentiation. Thus, we compared expression of the isoforms in non-differentiated myoblasts and on day 6 of the differentiation. The twenty-fold increase in the expression of mRNA of isoform A2 was observed after the differentiation (Fig. 1), demonstrating organism-like behaviour [7]. At the same time, there was less significant than for EEF1A2, yet statistically credible five-fold increase in EEF1A1 mRNA.

MicroRNAs play an important role during the differentiation of myoblasts and the development of pathologies of muscular and cardiac tissues [16]. To check a possible participation of microRNA in the regulation of expression of the isoforms during the differentiation of myoblasts, we analyzed *in silico* microRNA binding sites in 3'-UTR of mRNA EEF1A1/A2. mRNA of the eEF1A1 isoform contains mir-133, mir-543, mir-33 binding sites, and mRNA of EEF1A2 could contain binding sites of mir-744, mir-661 and mir-675. Noteworthy, unlike EEF1A2, 3'-UTR of EEF1A1 mRNA contains the binding site for mir-133, the crucial microRNA for the differentiation of myoblasts [16].

The correlation of the switch in the expression of microRNA with disordered expression of target proteins is one of the facts testifying for the existence of post-transcriptional control over these proteins. The quantitative PCR-analysis of the level of expression of all microRNAs, envisaged by the bioinformatic

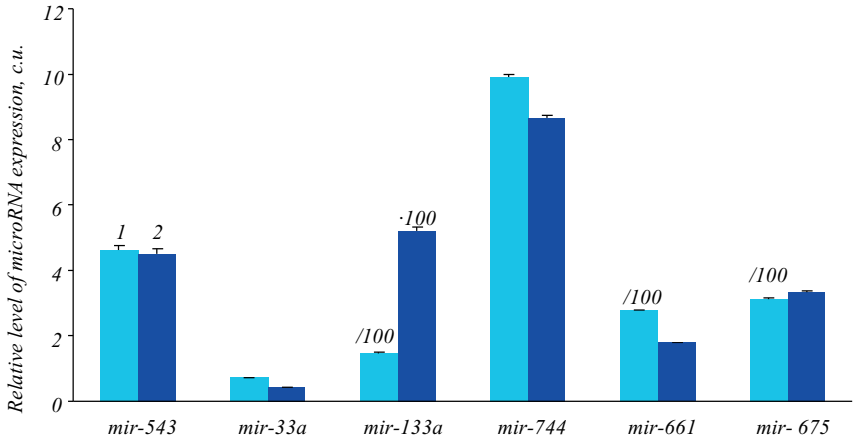

Fig. 2 The expression of microRNA with envisaged binding sites in 3'-NTS mRNA EEF1A1/A2 during the differentiation of myoblasts. The quantitative PCR-analysis of myoblasts in proliferating myoblasts (*1*) and differentiating myocytes (*2*)

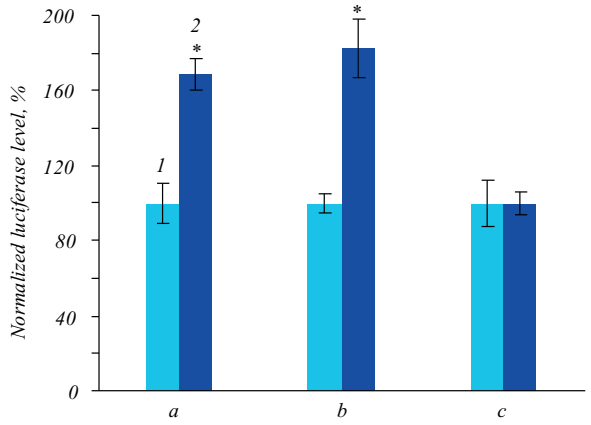

Fig. 3 The post-transcriptional control of the expression of isoforms of translation elongation factor eEF1 during the differentiation of myoblasts. Proliferating myoblasts (*1*) or differentiating myocytes (*2*) of LHCN were transfected by reported plasmids, containing the open reading frame of luciferase and 3'-UTR EEF1A1/A2: *a* – *pSICHECK* + EEF1A1 3'-UTR; *b* – *pSICHECK* + EEF1A2 3'-UTR; *c* – *pSICHECK-2*; *t*-Student's test. The level of luciferase was measured after 24 h of incubation; **p* < 0.05; *t*-Student's test

method, showed five hundred-fold increase in the mir-133 content (Fig. 2). Such significant increase could also cause the post-transcriptional blockage of EEF1A1 mRNA. Isoform A2 demonstrated regulation by two microRNAs, mir-744 and mir-661, the expression of which decreased during the differentiation by 13 and 36 %, respectively. The reduction in the content of these microRNAs correlates with the increase in the quantity of EEF1A2 mRNA during the differentiation (Fig. 1).

We used the method of reporter genes to prove the possibility of the post-transcriptional control of the switch of isoforms during the differentiation of myoblasts. Proliferating myoblasts and differentiated myocytes after six days of differentiation were transfected by a reporter plasmid, containing 3'-UTR of mRNAs coding for A1 or A2, respectively. The experimental results demonstrated almost two-fold increase in the level of luciferase with 3'UTR mRNA A2 (Fig. 3), which correlated with earlier obtained data about the increase in the quantity of A2 mRNA and the reduction of the level of expression of microRNA, which potentially regulate A2 (Fig. 1, 2). At the same time we detected some positive post-transcriptional regulation of expression of the reporter gene for A1 (Fig. 3).

Twenty-fold change in the A2 expression at the mRNA level and two-fold change in case of application of the reporter vector fused with 3'-UTR of A2 mRNA may testify for some contribution of the post-transcriptional regulation in the positive control of the expression of mRNA of isoform A2 during the differentiation of myoblasts.

Thus, the stimulation of the EEF1A2 mRNA expression during the differentiation occurs at both transcriptional and post-transcriptional levels. At the same time, the inhibition of the A1 isoform, highly expressed in all tissues [14], apparently requires a regulation which could be more powerful than microRNA. Indeed, even highly expressed mir-133 does not inhibit EEF1A1 expression in our experiments, which correlates with the literature data [17].

Conclusions. The results obtained demonstrate that the induction of EEF1A2 expression during myoblasts differentiation is controlled at both transcriptional and post-transcriptional levels. Besides, the increase in the expression of mRNA coding for the isoform A2 and the

reporter gene, containing 3'-UTR of mRNA coding for eEF1A2, correlates with the decrease in the expression of mir-744 and mir-661 possessing predicted binding sites in 3'-UTR of EEF1A2 mRNA. This fact suggests that these microRNAs participate in the post-transcriptional control of this isoform expression. Unexpected was the increase in the A1 isoform expression after the differentiation of myoblasts, which is not characteristic of this process in vivo. This could be explained by artificial conditions of differentiation, namely, the use of insulin as one of the main components of the "differentiation" medium. This hormone was shown to stimulate expression of mRNA, containing oligopyrimidine tract in 5'-UTR [18]. EEF1A1 mRNA belongs to this class of mRNA [19].

The obtained data contribute significantly to the understanding of the fundamental mechanisms of the differentiation of myoblasts and may help in further elucidation of the mechanisms of cancer development, as occurrence of the A2 isoform in unspecific tissues results in malignant transformation of cells [10].

Nevertheless, the negative regulation of eEF1A1 in muscular and nervous tissues remains an open issue and requires further investigation.

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Транскрипційний і посттранскрипційний контроль експресії eEF1A2 у процесі диференціації міобластів

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Резюме

У процесі постнатального розвитку в нейрональній і м'язовій тканинах відбувається зміна ізоформи А1 фактора елонгації трансляції на ізоформу А2. Перемикання експресії ізоформ є життєво важливою подією, оскільки мутантні миші, що містять делецію EEF1A2, гинуть на 28-й день після народження. Механізми інгібування А1 і стимуляції А2 протягом перших днів постнатального розвитку невідомі. Наявність сайтів зв'язування мікроРНК у 3'-нетрансльованих послідовностях мРНК ізоформ фактора елонгації трансляції передбачає існування посттранскрипційного контролю даного процесу. ***Мета****. Перевірити можливість посттранскрипційної регуляції експресії ізоформ А1 і А2 під час диференціації ім- морталізованих міобластів людини LHCN.* ***Методи****. Рівень експресії генів визначали методом кількісної ПЛР, наявність посттранскрипційного контролю детектували методом репортерних генів.* ***Результати****. Використовуючи як модель клітинну лінію іммортalізованих міобластів людини LHCN, показано індукцію ізоформи А2 фактора елонгації трансляції eEF1 при диференціації міобластів, наявність транскрипційного і посттранскрипційного контролю в даному процесі, а також потенційну участь мікро- РНК у процесі зміни експресії ізоформ.* ***Висновки****. Індукція ізоформи А2 протягом диференціації міобластів може відбуватися як на транскрипційному, так і на посттранскрипційному рівні.*

Ключові слова: eEF1A1, eEF1A2, іммортalізовані міобласти людини LHCN, диференціація, мікроРНК.

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Транскрипционный и посттранскрипционный контроль экспрессии eEF1A2 в процессе дифференциации миобластов

Резюме

В процессе постнатального развития в нейрональной и мышечной тканях происходит изменение экспрессии изоформы А1 фактора элонгации трансляции на экспрессию изоформы А2. Переключение экспрессии изоформ является жизненно важным фундаментальным процессом, поскольку мутантные мыши, содержащие делецию части гена EEF1A2, погибают на 28-й день после рождения. Механизмы ингибирования экспрессии А1 и стимуляции А2 в течение первых дней постнатального развития неизвестны. Наличие потенциальных сайтов связывания микроРНК в 3'-нетранслируемых последовательностях мРНК изоформ фактора элонгации трансляции предусматривает возможность посттранскрипционного контроля данного процесса. ***Цель****. Проверить возможность посттранскрипционной регуляции экспрессии изоформ А1 и А2 во время дифференциации иммортализованных миобластов человека LHCN.* ***Методы****. Уровень экспрессии генов определяли методом количественного ПЛР, наличие посттранскрипционного контроля детектировали методом репортерних генов.* ***Результаты****. Используя клеточную линию иммортализованных миобластов человека LHCN как модель, показана индукция изоформы А2 фактора элонгации трансляции eEF1 при дифференциации миобластов, наличие транскрипционного и посттранскрипционного контроля в данном процессе, а также потенциальное участие микроРНК в процессе изменения экспрессии изоформ.* ***Выводы****. Индукция изоформы А2 при дифференциации миобластов может происходить как на транскрипционном, так и на посттранскрипционном уровне.*

Ключевые слова: eEF1A1, eEF1A2, иммортализованные миобласты человека LHCN, дифференциация, микроРНК.

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