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TRANSCRIPTIONAL REGULATION OF *NOX* GENES EXPRESSION IN HUMAN BREAST ADENOCARCINOMA MCF-7 CELLS IS MODULATED BY ADAPTOR PROTEIN Ruk/CIN85

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NADPH oxidases are key components of redox-dependent signaling networks involved in the control of cancer cell proliferation, survival and invasion. The data have been accumulated that demonstrate specific expression patterns and levels of NADPH oxidase homologues (NOXs) and accessory genes in human cancer cell lines and primary tumors as well as modulation of these parameters by extracellular cues. Our previous studies revealed that ROS production by human colorectal adenocarcinoma HT-29 cells is positively correlated with adaptor protein Ruk/CIN85 expression while increased levels of Ruk/CIN85 in weakly invasive human breast adenocarcinoma MCF-7 cells contribute to their malignant phenotype through the constitutive activation of Src/Akt pathway. In this study, to investigate whether overexpression of Ruk/CIN85 in MCF-7 cells can influence transcriptional regulation of NOXs genes, the subclones of MCF-7 cells with different levels of Ruk/CIN85 were screened for NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2 as well as for regulatory subunit $p22^{Phox}$ mRNA contents by quantitative RT-PCR (qPCR). Systemic multidirectional changes in mRNA levels for NOX1, NOX2, NOX5, DUOX2 and $p22^{Phox}$ were revealed in Ruk/CIN85 overexpressing cells in comparison to control WT cells. Knocking down of Ruk/CIN85 using technology of RNA-interference resulted in the reversion of these changes. Further studies are necessary to elucidate, by which molecular mechanisms Ruk/CIN85 could affect transcriptional regulation of NOXs genes.

K e y w o r d s: NOX genes, transcriptional regulation, qPCR, MCF-7 cells, adaptor protein Ruk/CIN85.

Multiple experimental data from *in vitro* cell line studies and xenograft models as well as screening of primary tumor samples suggest that reactive oxygen species (ROS) act as second messengers in intracellular signaling pathways that can strongly influence tumor growth and survival, and support the oncogenic potential of cancer cells [1-3]. One of the main sources of ROS within cells is members of NADPH oxidase family (NOX/DUOX family), which include 7 homologs: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2 [4, 5]. ROS produced by NADPH oxidases at physiologically relevant concentrations in time- and space-dependent fashion mediate oxidative modifications of biomacromolecules thus regulating their biological activity [4, 6, 7]. Expression patterns of NOX enzymes and their accessory proteins in tumor cell lines and tumor samples are highly tissue-specific [5, 8]. Currently, it is known that growth factors and cytokines, integrin-dependent stimulation as well as hypoxia can induce changes in expression and activity of NADPH oxidases [5].

Among the important components of signaling networks involved in the control of cell physiology are adaptor/scaffold proteins [9]. Having modular structure, these proteins not only determine the formation and localization of signaling complexes but also control specificity, efficiency and the amplitude of signal propagation [10]. Adaptor/scaffold protein Ruk/CIN85 containing three SH3 domains, proline-rich region and C-terminal coiled-coil domain was shown to be a platform for signaling complexes formation involved in the control of fundamental cellular and signaling events as well as carcinogenesis [11, 12]. According to our previous results, the adaptor protein Ruk/CIN85 can form the intracellular

complex with adaptor protein Tks4 [13, 14], which functions as organizer subunit of NADPH-oxidase complex mediated by Nox1 [15, 16]. We have also revealed that ROS production by human colorectal adenocarcinoma HT-29 cells is positively correlated with adaptor protein Ruk/CIN85 expression [17]. Using expression vector encoding fluorescent sensor of hydrogen peroxide HyPer fused with adaptor protein Ruk/CIN85 and live cell fluorescence microscopy, the co-localization of Ruk/CIN85 and H_2O_2 generation was shown to take place at the edges of representative vesicular structures in transiently transfected MCF-7 cells [18]. In addition, it was demonstrated that high levels of Ruk_1/CIN85 contribute to the conversion of breast adenocarcinoma cells into a more malignant phenotype via modulation of the Src/Akt pathway [19], development of chemoresistance [20], constitutive activation of transcription factors HIF-1α [21] and NF-κB (unpublished data).

In this study, we demonstrate the potential of signaling adaptor Ruk/CIN85 to differentially influence *NOX* genes and NOX accessory gene *p22^{Phox}* expression using as a model human breast adenocarcinoma MCF-7 cells stably overexpressing different levels of Ruk/CIN85.

Materials and Methods

Materials. InnuSOLV RNA Reagent (Analytik Jena AG, Germany), First Strand cDNA Synthesis Kit (Thermo Scientific, USA), Luminaris Color HiGreen Low ROX qPCR Master Mix (Thermo Scientific, USA).

Cell culture. Cell line MCF-7 (human breast adenocarcinoma) and its sublines with Ruk/CIN85 overexpression D4, G4 and G10 [19], and G4 cells stably infected with lentivirus encoding Ruk/CIN85-specific shRNA [17] were maintained in DMEM (Gibco®) containing 10% fetal bovine serum (HyClone), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco®) at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were split 1:3-1:5 every 2-3 days at 70-80% confluency.

RNA extraction. Total RNA was extracted from a 3.5 cm cell culture Petri dish by using the innuSOLV RNA Reagent (Analytik Jena AG, Germany). Purification was carried out according to the manufacturers' instructions. The purified RNA samples were stored in RNAse-free water at – 80 °C. Quantification of RNA was performed in duplicate by spectrophometry at 260 nm. Additionally, absorbtion ratios at 260/230 nm and 260/280 nm were measured and the quality of RNA was suggested to be adequate at value 1.6-2. The quality of RNA samples was also ensured by electrophoresis in agarose gel followed by ethidium bromide staining, where the 18S and 28S RNA bands could be visualized under UV light.

cDNA synthesis. Two μg of total RNA were reverse transcribed in a final volume of 20 μl containing 1x reaction buffer, 20 pmole dNTP mix, 20 U RiboLock TM RNAse inhibitor, 0.2 μg random hexamer primer, and 40 U M-MuLV reverse transcriptase (Thermo Scientific, USA). The reaction mix was incubated at 37 °C for 60 min before the reverse transcriptase was inactivated by a 5 min incubation at 70 °C. cDNA was stored at –80 °C.

Real-time PCR amplification. All target transcripts were detected using quantitative real-time RT-PCR assays (non-specific fluorescent dyes that intercalate with any double-stranded DNA). YWHAZ was chosen as the endogenous control for data normalization. Primers and probes were chosen with the assistance of the computer program Primer Express (Applied Biosystems, Foster City, CA, USA). Then nucleotidic sequence was 'Blasted' against the dbEST and non-redundant databases to confirm the total gene specificity of the nucleotide sequences chosen. Each primer set was positioned in different exons of the gene in order to avoid amplification of contaminating genomic DNA. The nucleotide sequences are shown in Table.

PCR reactions were performed on qTOWER2.2 device (Analytik Jena AG, Germany) using Luminaris Color HiGreen Low ROX qPCR Master Mix (Thermo Scientific, USA). Real-time detection was performed using non-specific fluorescent dyes (SYBR Green) and reference dye ROX. The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min, then 30-45 cycles at an appropriate annealing temperature depending on the primer set (shown in Table) for 30 s. Amplification was followed by melting at 60-95 °C with the purpose to estimate the number of PCR products. PCR products were then run on a 2% agarose gel in order to confirm the presence of a single band of the expected size.

For normalization, *YWHAZ* gene was used as reference and data quantification was performed using ddCt method as described before [22].

Primer sequences for RT-PCR amplification

Gene	PCR product, bp	Primers
NOX1	108	5′-cacaagaaaaatccttgggtcaa-3′ 5′-gacagcagattgcgacacaca-3′
NOX2	550	5′-ggagtttcaagatgcgtggaaacta-3′ 5′-gccagactcagagttggagatgct-3′
NOX3	376	5′-ccatgggacgggtcggattgt-3′ 5′-gggggcagaggtaagggtgaagg-3′
NOX4	125	5′-gcaggagaaccaggagattg-3′ 5′-cactgagaagttgagggcatt-3′
NOX4	65	5′-tggtgttactatctgtattttctcagg-3′ 5′-agttgagggcattcaccaga-3′
NOX5	100	5′-gcaggagaagatggggagat-3′ 5′-cggagtcaaatagggcaaag-3′
DUOX1	97	5′-ttcacgcagctctgtgtcaa-3′ 5′-agggacagatcatatcctggct-3′
DUOX2	91	5′-acgcagctctgtgtcaaaggt-3′ 5′-tgatgaacgagactcgacagc-3′
p22^{phox}	317	5′-gtttgtgtgcctgctggagt-3′ 5′-tgggcggctgcttgatggt-3′
YWHAZ	168	5′-atccgccatgacaaaggagg-3′ 5′-ctggatgttctgacttgagacg-3′

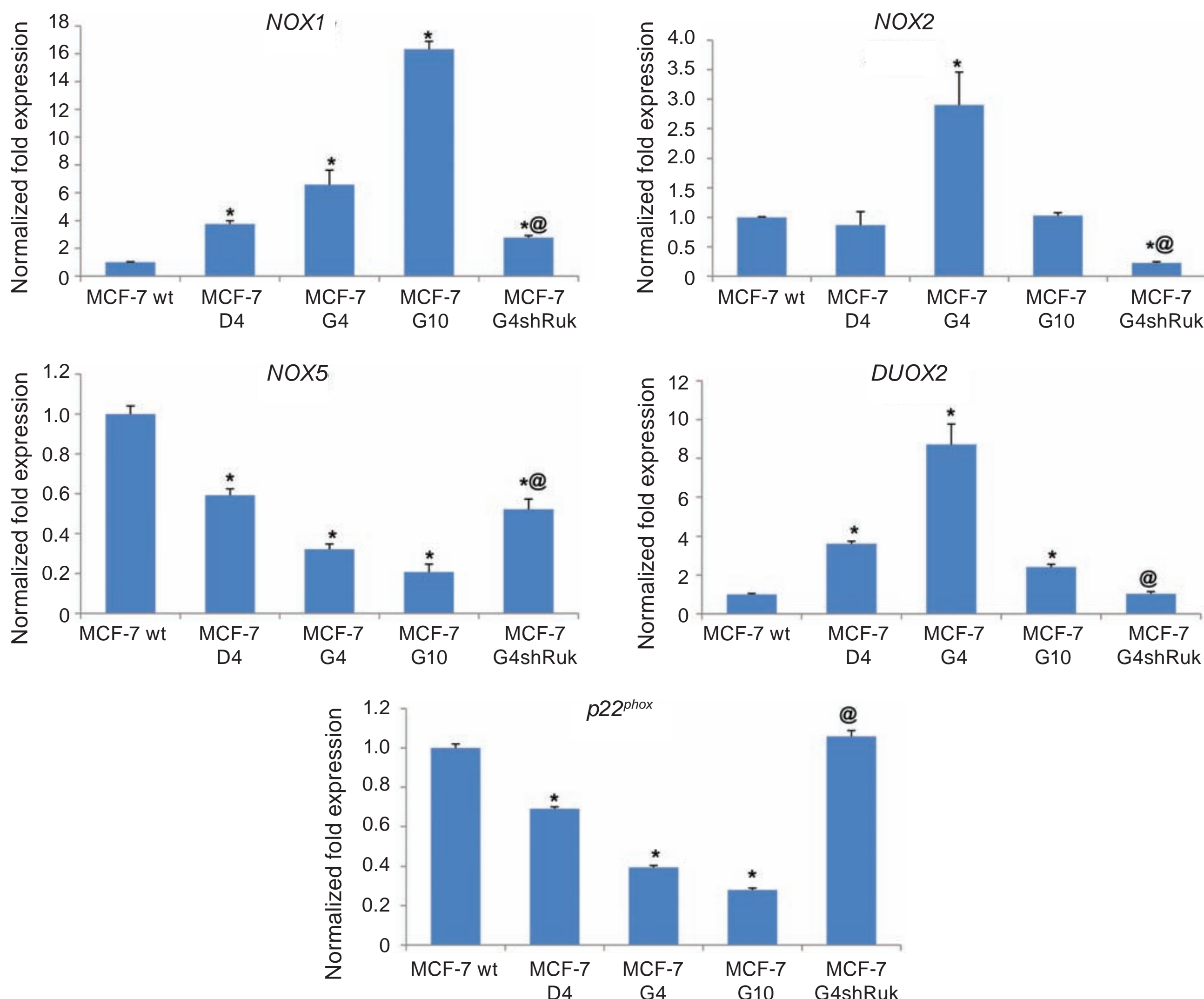
Results and Discussion

To study the possible influence of adaptor protein Ruk/CIN85 on transcriptional regulation of *NOX* genes, human breast adenocarcinoma MCF-7 cell line and its sublines with different levels of adaptor protein Ruk/CIN85 overexpression (D4<G10<G4) [18] as well as G4 cells, stably infected with lentivirus encoding Ruk/CIN85-specific shRNA (G4shRuk) [17], were used. The expression levels of *NOX* genes 1–5, *DUOX* genes 1–2 and gene for accessory protein *p22^{Phox}* were evaluated by qPCR relative to housekeeping gene *YWHAZ*. As shown in Figure, in cell lines studied the differential expression of *NOX1*, *NOX2*, *NOX5*, *DUOX2* and *p22^{Phox}* genes was detected. The specificity of the RT-PCR products was documented using high-resolution gel electrophoresis and resulted in a single product of desired length (NOX1: 108 bp; NOX2: 550 bp; NOX5: 100 bp; DUOX2: 91 bp; p22^{pho}x: 317 bp). In contrast, the expression of NOX3, NOX4 and DUOX1 was not revealed under the experimental conditions used or, in other case, was under the detection limit. Similar results regarding expression of *NOX1*, *NOX2* and *NOX5* genes in MCF-7 cells has been reported in [8] with the exception of *DUOX2* gene, which we were able to detect at low levels in WT MCF-7 cells.

As can be seen from Figure, considerable increase in mRNA content for *NOX1* and *NOX*2 genes was observed in subclones with high level of Ruk/CIN85 overexpression in comparison with WT MCF-7 cells and subclone D4 with low level of Ruk/CIN85 expression. Especially, NOX1 mRNA content was about 7 times higher in G4 cells and 16 times higher in G10 cells while NOX2 mRNA content was increased 3 times in G4 cells only. The level of *NOX1* expression was attenuated in G4shRuk cells. At the same time, the level of *NOX2* expression in G4shRuk cells was decreased below the level of *NOX2* characteristic of control cells. The most prominent increase in *DUOX2* mRNA content was detected in G4 cells while down-regulation of Ruk/CIN85 was followed by its restoration to control values. Interestingly, comparatively high levels of *NOX5* and *p22^{Phox}* expression were observed in WT MCF-7 cells. These values declined in MCF-7 subclones depending on Ruk/CIN85 expression and restored in G4shRuk cells. Since the expression level of a *p22^{phox}* gene inversely correlates with the expression levels of genes for catalytic subunits (*NOX1* and *NOX2*), it can be suggested the existence of optimal *p22^{phox}* expression level as a factor that ensures the formation of functionally active oxidase complexes. Our preliminary data obtained using CL assay (unpublished data) showed that G4 and G10 cells produce more ROS than control cells and D4 subclone that is in accordance with revealed changes in *NOX* genes expression. Taking into account the constitutive activation of NF-κB in Ruk/CIN85-overexpressing MCF-7 cells, there seems to be a positive feedback loop, in which NF-κB activation by NOX1/NOX2-derived O_2^- leads to further O_2^- production through induction of NOX enzymes [5].

In cell lines of epithelial origins, high levels of *NOX1* mRNA expression were observed in colorectal cell lines [8, 23], especially in HT-29 cells, for which the positive correlation between ROS production and expression of Ruk/CIN85 was demonstrated by us [17]. High levels of *NOX1*, *NOX2*, *DUOX1* and *DUOX2* genes expression were also detected in lung adenocarcinoma A549 cells, and ROS production by these cells was partially reduced by NADPH oxidase inhibitor diphenyleniodonium [24].

In conclusion, the studies presented herein demonstrate the potential of signaling adaptor Ruk/



*Ruk/CIN85 differentially modulates the expression of NOX genes and gene for accessory protein p22Phox in human breast adenocarcinoma MCF-7 cells that stably overexpress different levels of adaptor protein. Gene expression analysis was performed by qPCR relative to housekeeping gene YWHAZ. Data represent mean ±SD of three independent experiments. * $P_{(U)} < 0.05$ comparing to MCF-7 wt, @$P_{(U)} < 0.05$ comparing to MCF-7 G4. P values were calculated with Mann-Whitney U-test*

CIN85 to differentially influence *NOX* genes and NOX accessory gene *p22*^{Phox} expression in human breast adenocarcinoma MCF-7 cells that can be followed by increased production of ROS need to control physiological responses of tumor cells. To elucidate, by which molecular mechanisms Ruk/CIN85 could affect transcriptional regulation of NOXs genes, further studies are necessary.

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РЕГУЛЮВАННЯ ЕКСПРЕСІЇ ГЕНІВ *NOX* НА РІВНІ ТРАНСКРИПЦІЇ В АДЕНОКАРЦИНОМНИХ КЛІТИНАХ ГРУДНОЇ ЗАЛОЗИ ЛЮДИНИ ЛІНІЇ MCF-7 МОДУЛЮЄТЬСЯ ЗА УЧАСТЮ АДАПТЕРНОГО ПРОТЕЇНУ Ruk/CIN85

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NADPH оксидази є ключовими компонентами редоксзалежних сигнальних мереж, залучених до контролю проліферації, виживання та інвазії пухлинних клітин. Накопичено дані, які демонструють специфічні профілі та рівні експресії гомологів NADPH оксидаз (NOXs) та їхніх допоміжних протеїнів у лініях пухлинних клітин людини та первинних пухлинах, а також модулювання цих параметрів позаклітинними чинниками. Нашими попередніми дослідженнями виявлено, що продукування АФК аденокарциномними клітинами ободової кишки людини лінії HT-29 позитивно корелює з рівнем експресії адаптерного протеїну Ruk/CIN85, тоді як підвищені рівні експресії Ruk/CIN85 у слабо інвазивних аденокарциномних клітинах грудної залози людини лінії MCF-7 сприяють розвитку їхнього малігнізованого фенотипу через конститутивну активацію шляху Src/Akt. З метою з'ясування, чи впливає надекспресія Ruk/CIN85 у клітинах MCF-7 на регулювання транскрипції генів *NOXs*, був проведений скринінг вмісту мРНК NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, DUOX2 та регуляторної субодиниці $p22^{Phox}$ в субклонах клітин MCF-7 із різними рівнями експресії Ruk/CIN85 за допомогою кількісної полімеразної ланцюгової реакції (qPCR). Системні різноспрямовані зміни рівня мРНК для NOX1, NOX2, NOX5, DUOX2 та $p22^{Phox}$ було виявлено в клітинах MCF-7 із надекспресією адаптерного протеїну порівняно з контрольними клітинами дикого типу. Пригнічення експресії Ruk/CIN85 за допомогою технології РНК-інтерференції призводило до реверсії виявлених змін. З'ясування молекулярних механізмів, що опосередковують вплив Ruk/CIN85 на регулювання транскрипції генів *NOXs*, потребує подальших досліджень.

К л ю ч о в і с л о в а: гени *NOX*, регулювання на рівні транскрипції, qPCR, клітини MCF-7, адаптерний протеїн Ruk/CIN85.

РЕГУЛЯЦИЯ ЭКСПРЕССИИ ГЕНОВ *NOX* НА УРОВНЕ ТРАНСКРИПЦИИ В АДЕНОКАРЦИНОМНЫХ КЛЕТКАХ ГРУДНОЙ ЖЕЛЕЗЫ ЧЕЛОВЕКА ЛИНИИ MCF-7 МОДУЛИРУЕТСЯ С УЧАСТИЕМ АДАПТЕРНОГО ПРОТЕИНА Ruk/CIN85

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NADPH оксидазы являются ключевыми компонентами редоксзависимых сигнальных сетей, вовлеченных в контроль пролиферации, выживания и инвазии опухолевых клеток. Накоплены данные, демонстрирующие специфические профили и уровни экспрессии гомологов NADPH оксидаз (NOXs) и их вспомогательных протеинов в линиях опухолевых клеток человека и первичных опухолях, а также модулирование этих параметров внеклеточными факторами. Нашими предыдущими исследованиями выявлено, что продукция АФК аденокарциномными клетками ободочной кишки человека линии HT-29 положительно коррелирует с уровнем экспрессии адаптерного протеина Ruk/CIN85, тогда как повышенные уровни экспрессии Ruk/CIN85 в слабо инвазивных аденокарциномных клетках грудной железы человека линии MCF-7 способствуют развитию их малигнизированного фенотипа из-за конститутивной активации пути Src/Akt. С целью выяснения, влияет ли сверхэкспрессия Ruk/CIN85 в клетках MCF-7 на регуляцию транскрипции генов *NOXs*, был проведен скрининг содержания мРНК NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, DUOX2 и регуляторной субъединицы $p22^{Phox}$ в субклонах клеток MCF-7 с разными уровнями экспрессии Ruk/CIN85 с помощью количественной полимеразной цепной реакции (qPCR). Системные разнонаправленные изменения уровня мРНК для NOX1, NOX2, NOX5, DUOX2 и $p22^{Phox}$ были выявлены в клетках MCF-7 со сверхэкспрессией адаптерного протеина по сравнению с кон-

трольными клетками дикого типа. Угнетение экспрессии Ruk/CIN85 с помощью технологии РНК-интерференции приводило к реверсии выявленных изменений. Выяснение молекулярных механизмов, опосредующих влияние Ruk/CIN85 на регуляцию транскрипции генов *NOXs*, требует дальнейших исследований.

Ключевые слова: гены *NOX*, регуляция на уровне транскрипции, qPCR, клетки MCF-7, адаптерный протеин Ruk/CIN85.

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