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A New Mouse Strain with a Mutation in the *NFE2L2* (*NRF2*) Gene

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Abstract—Transcription factor NRF2 is involved in inflammatory reactions, maintenance of redox balance, metabolism of xenobiotics, and is of particular interest for studying aging. In the present work, the CRISPR/Cas9 genome editing technology was used to generate the NRF2$^{\Delta Neh2}$ mice containing a substitution of eight amino acid residues at the N-terminus of the NRF2 protein, upstream of the functional Neh2 domain, which ensures binding of NRF2 to its inhibitor KEAP1. Heterozygote NRF2$^{wt/\Delta Neh2}$ mice gave birth to homozygous mice with lower than expected frequency, accompanied by their increased embryonic lethality and visual signs of anemia. Mouse embryonic fibroblasts (MEFs) from the NRF2$^{\Delta Neh2/\Delta Neh2}$ homozygotes showed impaired resistance to oxidative stress compared to the wild-type MEFs. The tissues of homozygous NRF2$^{\Delta Neh2/\Delta Neh2}$ animals had a decreased expression of the NRF2 target genes: NAD(P)H:Quinone oxidoreductase-1 (*Nqo1*); aldehyde oxidase-1 (*Aox1*); glutathione-S-transferase A4 (*Gsta4*); while relative mRNA levels of the monocyte chemoattractant protein 1 (*Ccl2*), vascular cell adhesion molecule 1 (*Vcam1*), and chemokine *Cxcl8* was increased. Thus, the resulting mutation in the *Nfe2l2* gene coding for NRF2, partially impaired function of this transcription factor, expanding our insights into the functional role of the unstructured N-terminus of NRF2. The obtained NRF2$^{\Delta Neh2}$ mouse line can be used as a model object for studying various pathologies associated with oxidative stress and inflammation.

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Keywords: transcription factor NRF2, transgenic animals, inflammation, oxidative stress

INTRODUCTION

NRF2 (nuclear factor erythroid 2 related factor 2) is a transcription factor that controls expression of many genes products of which have antioxidant and anti-inflammatory properties. In the cytoplasm, NRF2 is associated with the protein KEAP1 (Kelch-like ECH-associated protein 1), which under normal conditions promotes permanent proteasomal degradation of NRF2 [1]. Simultaneously, KEAP1 serves as a redox-sensitive

Abbreviations: AOX1, aldehyde oxidase-1; ARE, antioxidant response element; CCL2, monocyte attractant protein; GOx, glucose oxidase; GSTA4, glutathione-S-transferase A4; HMOX1, hemoxygenase-1; KEAP1, Kelch-like ECH-associated protein 1; MEFs, mouse embryonic fibroblasts; Neh, Nrf2-ECH homology; NQO1, NAD(P)H: quinone oxidoreductase; NRF2, nuclear factor 2 related to erythroid factor 2; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule 1.

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regulator of NRF2 activity. When oxidizing agents and electrophiles enter the cell, KEAP1 undergoes thiol modification of its cysteine amino acid residues [2]. This modification keeps KEAP1 bound to the NRF2. Due to the lack of "vacant" negative regulators, the newly synthesized NRF2 accumulates in the cytoplasm and then moves to the nucleus. In the nucleus, NRF2 in complex with its coactivators, including small proteins of the Maf family, recognizes antioxidant response elements (ARE) sequences in the promoters of its target genes and triggers their transcription [3].

There are seven highly conserved Neh (NRF2-ECH homology)-domains in the NRF2 structure [4, 5]. The N-terminal part of the protein contains a Neh2 domain (amino acids (aa) 16-86) that includes two sequences known as DLG and ETGE motifs [6]. The NRF2 negative regulator KEAP1 binds to these sequences. KEAP1, being an adaptor protein for the E3 ubiquitin ligase complex Cullin 3 (Cul3), stimulates ubiquitinylation of seven lysine residues located in the Neh2 domain of NRF2 between the DLG and ETGE motifs and promotes proteasomal degradation of the latter [6, 7]. The domains Neh1 (aa 435-562), Neh4 (aa 112-134), Neh5 (aa 183-201), and Neh7 (aa 209-316) are responsible for interaction of NRF2 with its coactivators and corepressors [3, 8, 9]. The Neh6 domain (aa 338-388) contains two degron sequences that are recognized by the E3 ubiquitin-ligase β-TrCP [10, 11]. The C-terminal part of the protein contains the Neh3 domain (aa 562-605), which is responsible for recognizing ARE elements in the promoters of the NRF2 target genes and contains a VFLVPK motif that helps NRF2 to bind to the CHD6 helicase [12]. While NRF2 is complexly organized, this protein is partially disordered and its Neh2, Neh7, and Neh1 domains can only be structured for short periods of time [13].

NRF2 activates transcription of the genes of the 2nd phase of xenobiotic detoxification responsible for removal of the modified compounds from the cell. NRF2 also actively participates in the cell defense against electrophilic stress [14]. NRF2 also controls expression of the genes products of which are involved in glutathione biosynthesis, as well as enzymes that directly or indirectly neutralize reactive oxygen species (ROS): NAD(P)H:quinone oxidoreductase (NQO1), hemoxygenase-1 (HO1), catalase (CAT). Reduction of ROS, in turn, contributes to the cessation of inflammatory responses. When the *Nfe2l2* expression is decreased, inflammation increases, which can lead to organ and tissue damage [15]. Downregulation of the *Nfe2l2* expression in monocytes results in the increased production of pro-inflammatory cytokines [16]. Mouse models have shown increase in the ROS levels leading to prolonged oxidative stress after brain injury [17].

Gene knockouts are widely used to study functions of transcription factors. The *Nfe2l2* knockout mice lacking the NRF2 transcription factor were obtained more than a quarter of a century ago [18], and all subsequent experiments have been performed exclusively with this line. In these mice, a cistron from the lactose operon was inserted into the *Nfe2l2* gene, resulting in inability to synthesize functional mRNA and protein product. However, the use of knockout animals often results in the secondary effects that make it difficult to interpret the obtained results. It is likely that in the case of complete absence of any transcription factor, secondary effects may be due to the absence of its associated cofactors. It is important to create new models with mutations in the domains of transcription factors, rather than compromising integrity of the protein structure. Another problem with the gene deletion approach is possible removal of one or more noncoding RNAs found in the introns and exons.

In this work, a new mutant mouse line, $NRF2^{\Delta Neh2}$, carrying an 8-aa substitution at the N-terminus of NRF2, was obtained; embryonic fibroblasts (MEFs) were characterized, and changes in the mRNA levels of a number of NRF2 target genes in various tissues of these animals were determined. This mouse line can be used as a model object for studying embryonic mortality, various pathologies accompanied with oxidative stress and inflammation, as well as for studying aging processes.

MATERIALS AND METHODS

Animals and work with them. Animals were kept in individually ventilated cages (IVC system, TECNIPLAST S.p.A., Italy) with free access to food and water purified by reverse osmosis, in an environment free of specific pathogens, with light regime 12/12 (light on at 09:00), in rooms with air exchange rate more than 15 r/h, at 20-24°C, humidity 30-70%. Wood chips with minimal dust formation were used as bedding. Shelters and building materials for nests made of natural materials were used as enrichment of the environment. All materials supplied to the animals were sterilized by autoclaving.

Transgenic animal generation. The work with animals was approved by the local bioethics committee "Institute of Mitoengineering MSU" LLC, protocol #79 of April 28, 2015. Mutation in the *Nfe2l2* gene was introduced using CRISPR/Cas9 technology. The guide RNA (5'-GACTTGGAGAGTTGCCACCGCC) to the first exon of this gene was selected using the Feng Zhang laboratory (http://crispr.mit.edu/) service. The corresponding single-guide RNA (sgRNA) was produced by *in vitro* transcription of T7 (MEGAscript™ T7 Transcription Kit, Thermo Fisher, USA) on a matrix obtained by PCR amplification of a plasmid pX458 [19] with forward primer: 5'-TGTAATACGACGACTCAC TATATAGGGACTTGGAGTTGAGTTGCCACCGC CGTTTTTTTAGAGAGCTAGAAATAGC and reverse

primer: 5'-AGCACCACCGACACTCGGTGCCACT. The sgRNA was mixed with Cas9 mRNA (GeneArt™ CRISPR Nuclease, ThermoFisher) in a TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8).

Single-cell embryos for subsequent microinjection of the genetic construct were isolated from oviducts of female mice according to a standard protocol [20]. Female zygote donors were preliminarily hormonally stimulated according to the scheme described by Averina et al. [21] and fertilized by males of the corresponding lineage [21].

Microinjection of the genetic construct into the pronucleus of a fertilized oocyte was performed in an oocyte washing medium with phenol red, pH 7.4, without heparin (CooperSurgical, Inc., USA, USA), surrounded by vaseline oil (JSC Tatkhimfarmpreparaty, Kazan, Russia) on an inverted microscope (ECLIPSE Ti, Nikon, Japan) using two micromanipulators (TransferMan 4R, Eppendorf, Germany), according to the previously described protocol [20]. Zygotes after microinjection were incubated in an atmosphere of 5% CO_2 at 37°C in a Sequential Fert™ medium with phenol red (CooperSurgical, Inc.). After incubation, surviving embryos were implanted in oviduct funnel of surrogate females according to the standard protocol [20]. After birth and completion of lactation period, mice were genotyped using tip of the tail, according to the FELASA guidelines for genotyping transgenic rodents [22]. Tissue samples for genotype identification were frozen at −20°C until genotyping.

The resulting heterozygous mice ($Nrf2^{wt/\Delta Neh2}$) were crossed to the inbred line C57BL/6J to avoid potential effects of secondary mutations. Homozygous individuals of $Nrf2^{\Delta Neh2/\Delta Neh2}$ and wild-type $Nrf2^{WT/WT}$ mice were obtained from crosses between heterozygous pairs of $Nrf2^{wt/\Delta Neh2}$.

Mice genotyping. Alkaline extraction method was used to isolate genomic DNA from tissue samples [23] followed by purification of DNA by phenol-chloroform extraction. An Encyclo Plus PCR kit (Evrogen, Russia) with 50 ng of genomic DNA was used for genotyping. PCR1 with primers mNrf-F476 (5'-GCAG GCTATCTCTCCTAGTTCT) and mNrf-R668 (5'-CG GCTTCTTGGCACAG) and PCR2 with primers mNrf-F476 and mNrf-R1153 (5'-GACAGGCGTGATC TTACAG) were performed on the DNA template. PCR regime: 95°C for 5 min, followed by 35 cycles (95°C for 25 s, 60°C for 25 s, 72°C for 25 s). PCR products were analyzed electrophoretically in a 1.5% agarose gel.

Mouse embryonic fibroblasts (MEF). Embryos were isolated at 10-14th day post coitus from heterozygous pregnant females ($Nrf2^{wt/\Delta Neh2}$) crossed with a male heterozygote. MEF isolation was performed according to the method described in [24]. The cells were cultured on a Dulbecco's modified Eagle medium (DMEM) (PanEco, Russia).

RNA isolation, reverse transcription, and real-time PCR. Total cellular RNA was isolated using a QuickRNA MiniPrep reagent kit (ZymoResearch, USA) according to the manufacturer's protocol. RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA), RNA quality was confirmed electrophoretically. Reverse transcription was performed using a SuperScript III kit (ThermoFischer, USA) as described previously [25] for subsequent analysis by real-time PCR with an EvaGreen I intercalating dye (Syntol, Russia). The following primers were used for real-time PCR: *Hmox1* (forward: 5'-CACGCATATATATACCCGCTACC; reverse: 5'-TCATCTCCAGAGAGTGTTCATTCG); *Nqo1* (forward: 5'-GTCCTCCATCAAGATTCG; reverse: 5'-GC TAACTGCTAACTGCTAACTGCTAA); *Aox1* (forward: 5'-CATAGGTCAGGTTGAAGGTGAAGGT; reverse: 5'-GGCAGGAATCTTGTATTGG); *Gsta4* (forward: 5'-AGCAACATTCCTACAATTAAGAAGT; reverse: 5'-TC CTGACCACCACCTCAACATAG); *Vcam1* (forward: 5'-CCCTCCACACAAACCAAGCC; reverse: 5'-CCATTC CAGTCACTTCAACG); *Il-6* (forward: 5'-ACCGCTA TGAAGTTCCTCTCTCTC; reverse: 5'-CTCTGTGAA GTCTCCTCCTCTCC); *Cxcl8* (forward: 5'-ACTTCAA GAACATCCAGAGC; reverse: 5'-CTTTCCAGGTCA GTTAGTTAGCC). Nucleotide sequences of primers for *Ccl2* and reference genes *Rpl32* and *Gapdh* are given in Ref. [26].

Western blot. MEFs were lysed in a hot SDS buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 50 mM DTT, 0.01% bromophenol blue) for 5 min at 94°C. Proteins were separated by electrophoresis using 12% SDS-PAGE, transferred to a PVDF membrane (Bio-Rad, USA), and sequentially incubated with rabbit antibodies against NRF2 (Invitrogen, USA) and horseradish peroxidase-labeled goat anti-rabbit immunoglobulin secondary antibodies (Sigma Aldrich, USA). A West Dura Extended Duration Substrate (Thermo Fisher) was used as a substrate for peroxidase, and images were acquired using a ChemiDoc gel documentation system (Bio-Rad).

Cytotoxic test. Resazurin test was performed according to the standard protocol as described previously [25]. Experiments to study the effect of oxidative stress on survival of MEFs were performed when cells were exposed to 250 μM H_2O_2 (Ecotex, Russia) and 3 units/ml glucose oxidase (GOx) (Sigma-Aldrich®, USA) for 3 h, n = 3.

DNA sequencing. DNA sequencing was performed using an ABI PRISM® BigDye™ Terminator reagent kit v. 3.1 followed by analysis of reaction products on an Applied Biosystems 3730 DNA Analyzer.

Statistical analysis. The results of crosses between mice were analyzed using the χ^2 criterion. Differences in the levels of gene expression between groups, as well as in survival of MEFs, was determined using the unpaired Student's *t*-test.

RESULTS

Transgenic mice with a mutation in the *Nrf2* gene. Using CRISPR/Cas9 technology and guide RNA corresponding to the first exon of the *Nfe2l2* gene, a female F0 mouse was made with a 284 nt deletion affecting the boundary of the first exon and intron of the *Nfe2l2* gene (Fig. 1). To eliminate the influence of possible secondary mutations caused by the nonspecific effect of genomic editing, sequential crosses of heterozygotes with inbred mice of the C57BL/6J line were performed for ten generations. Heterozygote crosses then produced homozygous animals, as well as wild-type and heterozygous animals of different sexes.

The observed deletion in the region affecting the splicing site (5'-CAG|GTGCTGCCC) between the first exon and intron of the *Nfe2l2* gene could, theoretically, prevent splicing of the *Nfe2l2* pre-mRNA and lead to the subsequent degradation of this transcript. Thus, one would expect to obtain an animal knockout of *Nfe2l2*. However, cDNA sequencing of the *Nfe2l2* gene revealed deletion of the codon encoding 8 aa residues at the 3'-end region of the first exon (PPGLQSQQQ), which were replaced by two aa residues (RW) formed during translation of the non-coding region of the first intron (Fig. 1b). At the same time, rest of the NRF2 cDNA remained unchanged. Based on these data, we concluded that alternative splicing occurs in the obtained NRF2$^{\Delta Neh2}$ mouse line due to activity of the hidden donor splice site (5'-TGG|GTGGGGGAGGC) in the first intron of the *Nfe2l2* gene. Although we had not obtained the *Nfe2l2* knockout mouse, work on this unique mouse model with a mutation in this gene was continued.

NRF2$^{\Delta Neh2/\Delta Neh2}$ homozygous mice have increased embryonic lethality. A total of 22 wild-type (37%), 34 heterozygotes (57%), and 3 homozygotes (5%) were obtained from the crosses between heterozygous mice.

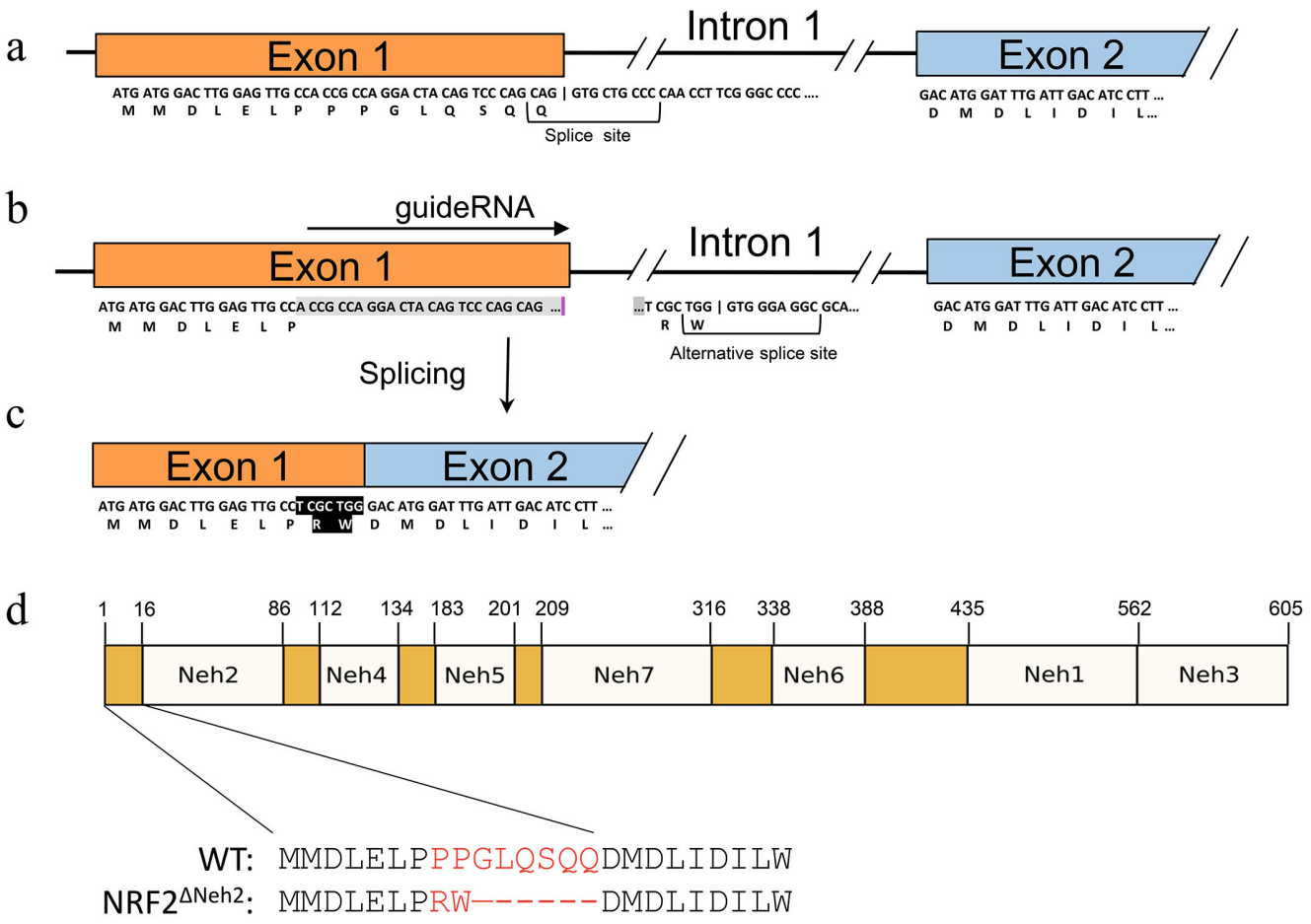

Fig. 1. Structure of *Nfe2l2* gene in wild-type and in the NRF2$^{\Delta Neh2}$ mice. a) WT pre-mRNA sequence of *Nfe2l2* gene; b) pre-mRNA sequence of the *Nfe2l2* gene with deletion obtained using CRISPR/Cas9 and guide RNA (gRNA). The deleted region of the gene is marked in gray, and alternative splicing site in the first intron of the gene is also depicted; c) sequence of the mature spliced mRNA of the *Nfe2l2* gene with the introduced mutation (substitutions are marked in black); d) domains of the NRF2 protein and comparison of the aa sequences between NRF2$^{\Delta Neh2}$ and WT. The mutation is located at the N-terminus of NRF2 and represents deletion of 8 aa (PPGLQSQQQ) substituted by two aa (RW) that were translated from the non-coding region of the first intron. The mutation affects positions 8-15, which are adjacent to the Neh2 domain responsible for binding of NRF2 to its negative regulator KEAP1.

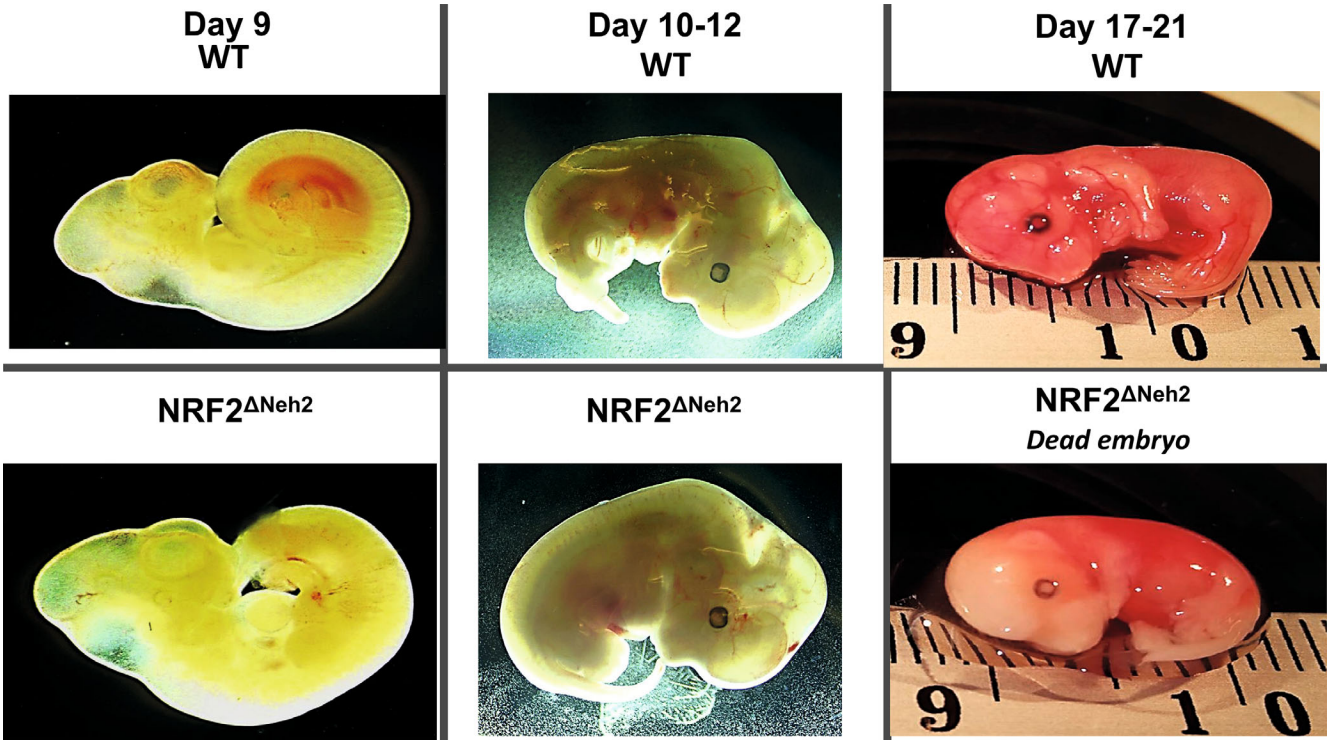

Fig. 2. Representative photographs of embryos from the wild-type (WT) and homozygous mice with mutation in the *Nfe2l2* gene ($NRF2^{\Delta Neh2}$). Embryos obtained by crossing heterozygotes at days 9, 10-12, and 17-21 were analyzed, and $NRF2^{\Delta Neh2}$ homozygotes show signs of anemia.

The number of genotypes obtained was not consistent with the Mendelian type of inheritance ($p = 0.001$ by χ^2 test), leading to assumption of increased embryonic lethality of homozygotes. Indeed, analysis of embryos obtained on days 9, 11, and 17-21 after conception showed signs of anemia and death in some homozygous embryos (Fig. 2). A total of 55 embryos were analyzed, of which 12 were wild-type (22%), 29 heterozygotes (53%), and 14 homozygotes (25%) ($p = 0.81$ by χ^2 test).

MEFs from homozygous $NRF2^{\Delta Neh2/\Delta Neh2}$ mice have reduced resistance to oxidative stress. Oxidative stress in MEFs was induced by addition of 250 μM H_2O_2 or 3 units/ml glucose oxidase (GOx) for 3 h. When H_2O_2 was used to induce oxidative stress, MEFs from the homozygous mutant mice had slightly lower survival rates compared to the cells from the wild-type mice (Fig. 3). Additionally, when GOx was added, survival rate of the MEFs from the wild-type mice was 50% and survival rate of the MEFs from homozygous mutant mice was 30%. This suggests that the MEFs from the animals with mutation in the *Nrf2* gene are less resistant to oxidative stress caused by addition of GOx compared to the wild-type cells.

MEFs of homozygous $NRF2^{\Delta Neh2/\Delta Neh2}$ mice have reduced expression levels of the *Nfe2l2* mRNA and its target genes. Homozygous MEFs with mutation in the *Nfe2l2* gene exhibited decrease in the expression of *Nfe2l2* mRNA and its target genes, as well as increase in the mRNA levels of the inflammation marker *Ccl2* (Fig. 4a). Interestingly, when analyzing the same sample of MEFs, the amount of *Nfe2l2* mRNA in the $NRF2^{\Delta Neh2}$ mice was approximately 10 times lower than in the WT mice, while the amount of NRF2 protein showed almost no difference (Fig. 4, b and c).

Tissues of $NRF2^{\Delta Neh2/\Delta Neh2}$ homozygous mice have reduced levels of the *Nfe2l2* mRNA and some of its target genes, while expression of the inflammatory markers

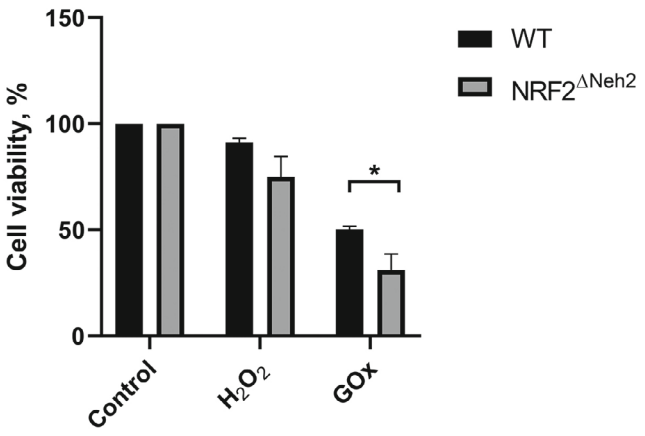

Fig. 3. MEFs from the homozygous mutant animals ($NRF2^{\Delta Neh2}$) have greater sensitivity to the GOx-induced oxidative stress than the MEFs from the wild-type (WT) animals. Cell death was induced by hydrogen peroxide (250 μM H_2O_2) or glucose oxidase (3 u/ml GOx). Fibroblast viability was measured by resazurin test. The data are presented as a mean +/– SEM, n = 3, * $p < 0.05$, when compared to WT by unpaired Student's *t*-test.

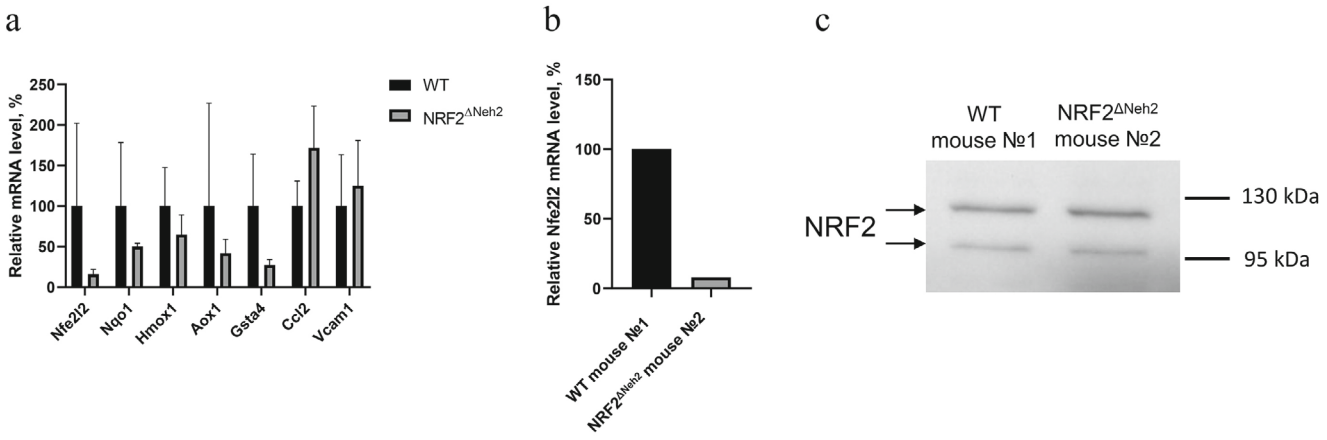

Fig. 4. Expression levels of *Nfe2l2*, its target genes, and inflammatory marker genes in the MEFs of NRF2 homozygotes$^{\Delta Neh2/\Delta Neh2}$ (NRF2$^{\Delta Neh2}$) and wild-type$^{NRF2wt/wt}$ (WT) mice: a) real-time PCR data of relative mRNA levels in the MEFs of WT and homozygous NRF2$^{\Delta Neh2}$ mice. *Hmox1*, heme oxygenase-1, NRF2 target; *Nqo1*, NAD(P)H:quinone oxidoreductase-1, NRF2 target; *Aox1*, aldehyde oxidase-1, NRF2 target; *Gsta4*, glutathione-S-transferase A4, NRF2 target; *Ccl2*, monocyte chemoattractant protein 1; *Vcam1*, vascular cell adhesion molecule 1. The mRNA level for WT was taken as 100%. The data are presented as a mean +/– SEM, n = 4. b) Relative amount of *Nfe2l2* mRNA in the MEFs of a mouse #1 (WT) and mouse #2 (NRF2$^{\Delta Neh2}$). c) Western blot of the NRF2 protein in the MEFs of mouse #1 (WT) and mouse #2 (NRF2$^{\Delta Neh2}$).

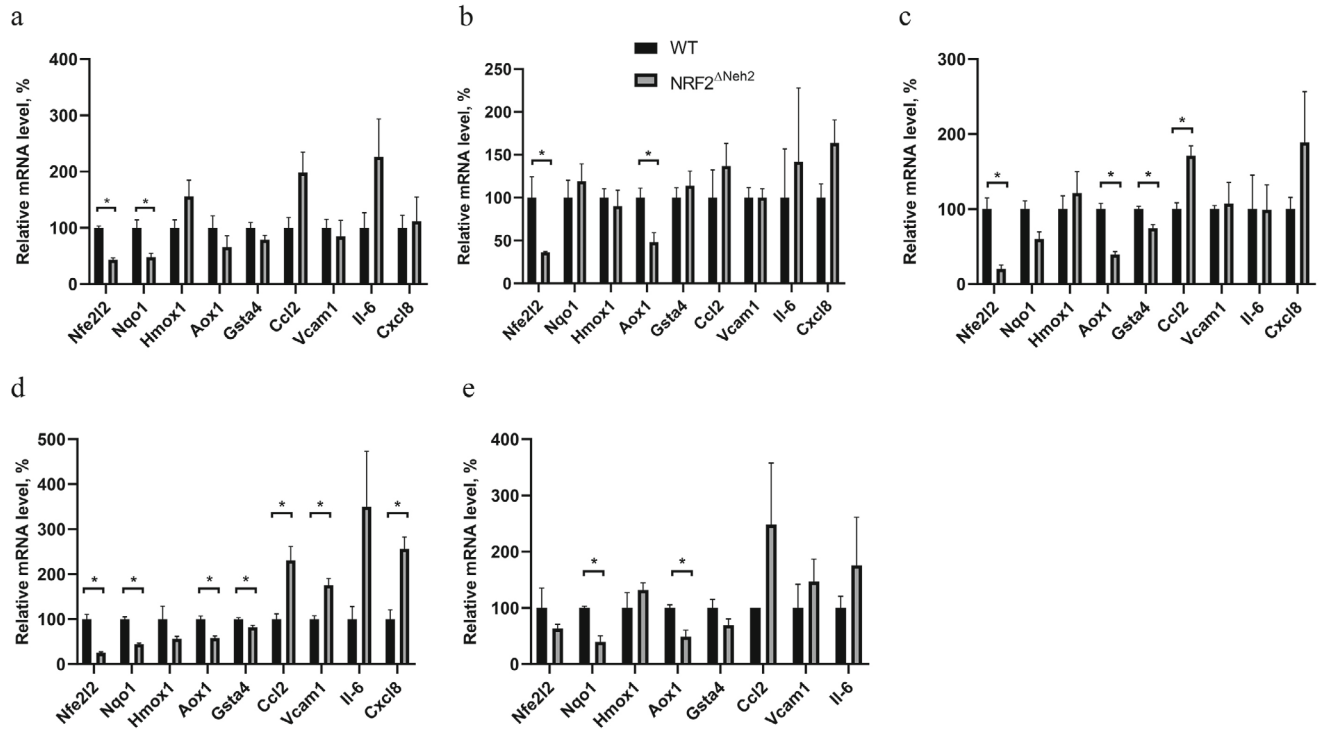

Fig. 5. Expression levels of NRF2, its target genes, and inflammatory marker genes in the tissues of wild-type (WT) animals and animals homozygous for the mutation in *Nfe2l2* (NRF2$^{\Delta Neh2}$). Real-time PCR results showing relative amounts of mRNA in: a) liver, b) brain, c) heart, d) kidney, and e) tibialis anterior muscle of the NRF2$^{\Delta Neh2}$ and WT mice. *Hmox1*, hemoxygenase-1, target of NRF2; *Nqo1*, NAD(P)H:quinone oxidoreductase-1, NRF2 target; *Aox1*, aldehyde oxidase-1, NRF2 target; *Gsta4*, glutathione-S-transferase A4, NRF2 target; *Ccl2*, monocyte chemoattractant protein 1; *Vcam1*, vascular cell adhesion molecule 1; *Il-6*, interleukin 6; *Cxcl8*, interleukin 8, chemokine. The mRNA level for WT was taken as 100%. The data are presented as a mean +/– SEM, n = 3. * $p < 0.05$ when compared to the WT by unpaired Student's *t*-test.

***Ccl2*, *Vcam1*, and *Cxcl8* is upregulated.** A twofold decrease in the expression of the *Nfe2l2* mRNA and its target *Nqo1*, and slight decrease in the expression of two other NRF2 targets, *Aox1* and *Gsta4*, were observed in the livers of homozygous mutant mice (Fig. 5a). There was also a twofold increase in the amount of the *Ccl2* gene mRNA, one of the markers of inflammation, in the liver of mutant animals. In the brains of homozygous mutant animals, the expression of *Nfe2l2* and its target gene *Aox1* decreased twofold; the amount of mRNA of other genes was almost unchanged (Fig. 5b). In the heart of homozygous mice, we observed a signif-

icant decrease in the amount of *Nfe2l2* mRNA and its target gene *Aox1* and slight decrease in the expression level of *Nqo1* (Fig. 5c). Also, in the heart of NRF2$^{\Delta Neh2}$ animals, there was a slight increase in the mRNA level of *Ccl2*. In the kidneys of the NRF2$^{\Delta Neh2}$ mice, there was a significant decrease in the expression level of *Nfe2l2*, *Nqo1*, and *Aox1* and increase in the mRNA amounts of the inflammation marker genes *Ccl2*, *Vcam1*, and *Cxcl8* (Fig. 5d). In the tibialis anterior muscle of the homozygous mice, there was decrease in the amount of *Nfe2l2*, *Nqo1*, and *Aox1* mRNAs, slight decrease in the *Gsta4* expression, and increase in the amount of mRNA for the inflammation marker *Ccl2* (Fig. 5d). Surprisingly, there was no significant change in the expression level of *Hmox1* in any of the tissues examined. Measurement of the relative mRNA level of the chemokine *Cxcl8* in the tibial muscle, as well as of the mRNA of the cytokines *Tnf* and *IL-1β* in all tissues was not possible due to the high values of threshold cycles (Ct > 35 cycles) in real-time PCR.

Thus, in the tissues of the NRF2$^{\Delta Neh2}$ homozygous mice expression levels of both *Nfe2l2* itself and some of its targets decreased, and at the same time the expression level of some inflammatory markers increased.

DISCUSSION

We have created the mice with mutation in the gene encoding the NRF2 transcription factor (Fig. 1). The mutation involves 8 amino acid residues located in the first exon of the N-terminal part of the NRF2 sequence at positions 8-15 (PPGLQSQQQ), which are replaced by two aa (RW). The N-terminal part of NRF2 contains the Neh2 domain responsible for binding of NRF2 to its negative regulator KEAP1, but amino acid residues located at positions 1-15 are usually not included in the Neh2 domain because they are not conserved among the NRF2 homologous proteins [27, 28]. However, in the work of MacMahon et al. [11] it was shown that deletion of amino acid residues 1-16 leads to the slight increase in the half-life of NRF2, and this effect is not associated with the impaired protein ubiquitination. In this study, it was shown that despite the fact that the introduced mutation affects a non-conserved region of the NRF2 amino acid sequence, it, nevertheless, affects protein function, which is manifested in the mutant animals as increased embryonic mortality, decreased MEF survival, reduced amounts of *Nfe2l2* mRNA and some of its targets, and increased expression of the inflammatory markers in the MEFs and some organs of the NRF2$^{\Delta Neh2}$ homozygotes.

It is known that the decreased NRF2 levels lead to the decreased cell and tissue resistance to oxidative stress. Thus, hyperoxia caused more pronounced lung damage in the *Nfe2l2* knockout mice compared to the WT [29]. In these same animals, exposure to cigarette smoke and diesel exhaust caused increase in the 8-oxo-7,8-dihydro-2'-deoxyguanosine levels, indicating oxidative DNA damage [30, 31]. In addition, the increased oxidative damage has been observed in the knockout mice upon exposure to allergens that lead to airway inflammation [32], a similar effect has been observed in sepsis [33]. It was previously shown that MEFs from the *Nfe2l2* knockout mice have an increased sensitivity to oxidative stress induced by diquat dibromide [34], as well as organic and inorganic peroxides [35]. Consistent with the above observations, we also observed decreased survival of the *Nfe2l2* mutant MEFs in our work (Fig. 3).

An unexpected result of our work was observation that mutation in the N-terminal region of NRF2 leads to increased embryonic lethality (Fig. 2). Part of the homozygous embryos had signs of anemia, which may be a consequence of the impaired erythrocyte production, erythropoiesis. It is interesting to note that the study of transcription factor NRF2 began due to the discovery of its role in erythropoiesis, which is reflected in its name [nuclear factor (erythroid-derived 2)-like 2]: NRF2 was discovered as a protein that recognizes the promoter region of the beta-globin gene [36]. However, no erythropoiesis-related abnormalities were observed in the generated NRF2 knockout mice, nor did they exhibit anemia, embryonic lethality, or reduced fertility [17]. It is conceivable that the NRF2 mutation could affect erythropoiesis in the homozygous mice in other ways: for example, by disrupting the ability of NRF2 to regulate expression of the genes responsible for heme biosynthesis, a key component of hemoglobin (see Ref. [37]). Nevertheless, the exact causes and mechanisms of embryonic death remain unknown.

The MEFs of NRF2$^{\Delta Neh2}$ homozygous mice showed decreased mRNA levels of *Nfe2l2*, as well as decreased mRNA levels of most of its targets (Fig. 4a). This decrease was also observed in the tissues of homozygous animals (Fig. 5) suggesting that the NRF2 transcriptional activity is impaired in these mice. Similar changes in the gene expression profile have been previously described in various tissues of the *Nfe2l2* knockout mice [38-41]. Interestingly, although the *Nfe2l2* mRNA levels were downregulated in the MEFs of the NRF2$^{\Delta Neh2}$ homozygotes compared to the WT, the amount of protein on Western blot was almost indistinguishable (Fig. 4, b, and c). This could probably be a consequence of the increased lifetime of the NRF2$^{\Delta Neh2}$ protein, as described by MacMahon et al. [11], however, this assumption requires further verification.

NRF2 controls the expression of genes that regulate inflammatory response, and its activation in various *in vitro* and *in vivo* models generally reduces inflammation, including downregulation of the chemokine *Ccl2* and the vascular cellular adhesion molecule *Vcam1* [42-44].

The NRF2$^{\Delta Neh2}$ mice showed increased expression of these genes in most tissues, indicating that this mouse line can be used as a model for excessive inflammatory activation, particularly in renal tissues where the greatest changes in gene expression were observed (Fig. 5d).

NRF2 also controls genes that regulate antioxidant response of the cells including diaphorase *Nqo1* and genes encoding important glutathione biosynthesis enzymes, such as glutathione transferases and glutamate-cysteine ligases. The levels of *Nqo1* and *Gsta4* mRNAs were found to be decreased in various tissues of the NRF2$^{\Delta Neh2}$ mice (Fig. 5), which suggests potential impairment of the redox balance and possible development of oxidative stress in these animals. However, this issue was not investigated in this study.

Thus, in our work, we obtained a new mutant mouse line with substitution of 8 aa at the N-terminal region of NRF2, which resulted in partial disruption of the functions of this transcription factor and increased embryonic mortality. The obtained mouse line can be used as a model object to study various developmental pathologies associated with the impaired NRF2 functions.

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Conflict of interest. The authors of this work declare that they have no conflicts of interest.

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