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Research Article

Impact of *NOX4* Knockout by CRISPR/Cas9 on the MCF-7, HCA-7 and UM-RC-6 Cancer Cells

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Background: The second most common cause of mortality is cancer. Increased NOX4 expression is linked to cancer development and metastasis. However, the significance of NOX4 in cell growth and assault, remains unclear.

Objective: This study aimed to evaluate the effect of *NOX4* knockouts in MCF7, UM-RC-6, HCA-7 cell lines.

Materials and Methods: The *NOX4* gene was knocked out in MCF7, UM-RC-6, and HCA-7 cell lines through using CRISPR Cas-9 genetic engineering techniques. After transfection, the CRISPR Cas-9 cassette, the T7 endonuclease I, qPCR, and western blotting assay detected the *NOX4* knockouts. MTT and Annexin assessed the percentage of cell proliferation and apoptosis. Real-time PCR was used to measure the expression of pro- and anti-apoptotic genes.

Results: Occurrence of *NOX4* gene knockout in the examined cell lines, was confirmed by q-PCR and Western blot ($P<0.001$). The *NOX4*-deleted cell lines with increased sub-G1 caused lowered cell proliferation and population at S / G2/ M phases. *In Vitro*, *NOX4* silencing caused lowered expressions of anti-apoptosis genes *BCL-2* and *SURVIVIN* ($P<0.0001$), leading to increased tendency of apoptosis in the cell lines ($P<0.0001$) of the apoptotic genes *BAX*, *P53*, *FAS*. Additionally, the MTT and Annexin results of the target gene *NOX4* knockout inhibited proliferation, increased mortality rates ($P<0.01$), and increased apoptosis.

Conclusion: The findings of this study indicate that using *NOX4* as a target can have therapeutic value for creating potential treatments against breast, colorectal, and kidney cancers which shows a need for a deeper understanding of the biology of these cancers with direct clinical outcomes for developing novel treatment strategies.

Keywords: CRISPR-Cas9, Cancer, HCA-7, MCF7, *NOX4* Knockout, UM-RC-6

1. Background

Cancer ranked as the second biggest cause of mortality globally, behind cardiovascular illnesses, causing societal and economic harm (1). Mutations are occurring in people's genomes more than ever because of excessive biological progressions (2). Colon cancer is the world's 3rd most common cancer in males and the 2nd most frequent disease in females (3). About 5% of colorectal cancer patients have an additional primary cancer (4). 30% of cancer detection cases in women are related to breast cancer, making it the most frequent cancer type among women and a 15% mortality rate (5). According to research, breast cancer patients over 50 are also more likely to acquire colorectal cancer (6). As cytoplasmic signaling factors, reactive oxygen species (ROS) are participated in a range of biological processes (7). The formation of reactive oxygen species (ROS) is controlled by NADPH-oxidases (8). The physiological actions of NADPH oxidases are diverse, and they contribute to cell division, kidney function regulation,

microorganism immune response, but their excessive expression is seen in cancers (9). NOX4 is a generated NOX isoform that is essential for H_2O_2 production and belongs to the NOX class (10); recent studies have reported that *NOX4* is involved in many cancers (12, 13). Although the mechanisms with which *NOX4* regulates proliferation, migration, and survival of cancer cells are not clarified, a few studies show that NOX 4 causes cell proliferation through cell cycle regulation and apoptosis inhibition (14, 15). With advances in molecular biology, genome editing technologies can change genomes and observe functional changes caused by genetic modulation. Multiple methods have been used for treating malignancies that affect the normal cells of the body. Nevertheless, further attempts are needed to develop cancer and oncology treatment methods using molecular biology (16). Recently the Cas 9 protein system associated with the Clustered Regularly Interspaced The national cancer institute has used short Palindromic Repeats (CRISPR) to reduce the deaths caused by cancer as a novel therapeutic and powerful technology with high accuracy and efficiency for treating cancer (17).

2. Objectives

This study is one of the newest studies using the CRISPR-Cas9 system to knockout several cancer cells. It compared the effects of deleting *NOX4* on the cellular metabolism of cells to determine the effect on relevant cancer progression.

3. Materials and Methods

3.1. Materials and Cell Line Acquisition

In this study, we acquired the MCF-7 (CVCL_0031) breast cancer, UM-RC-6 (CVCL_2741) kidney cancer, and HCA-7 (AddexBio C0009003) colon cancer cell line from Iran's Pasteur Institute's national cell collection.

3.2. Target Design and Cloning

3.2.1. SgRNA Designing

The CRISPR/Cas9 Targeting Online Predictive model was used to generate the sgRNAs (http://crispr.mit.edu/). The nucleotide sequence was obtained from the GenBank dataset of the National Center for Biotechnology Information (NCBI, National Biosciences, Inc., Plymouth, MN).

3.2.2. CRISPR/Cas9 Knockdown of NOX4

The *NOX4* gene's activity was disrupted using the CRISPR/Cas9 technology. The sgRNA sequence targeting *NOX4* was 5› AAAAGGGG TTTTCCCCAAAA 3›. To analyses the activity of sgRNAs in vector-treated cells, both the forward and reverse primers were designed for PCR upstream of the first and downstream of the second sgRNA. Forward and reverse primers were designed for positions 46527 to 46549 (forward) and 47314 to 47335 (reverse). With the function of sgRNAs, the PCR product had a 550 bp band and, in the negative control, 808 bp. The forward primer sequence was GGGTCCAA CACCACATAAATGTG, and the rivers primer sequence was GAGTCCGTATCTCCGAAACTCA. PCR was performed to determine the presence of SgRNA in the pSpcas9 (BB) -2A-Poor (PX459) V2.0 crystalline vector. Also, individually targeted small interfering RNAs (siRNAs) were transfected into Colon cancer cell lines, with a nontargeting siRNA (control) serving as a negative control. siRNAs of the following sequences were bought from Pishgaman Gene Transfer Company (GTP Company, Iran).

siRNA1 sense:
5′-GCCUCAGCAUCUGUUCUUATT-3′,

siRNA1 antisense:
5′-UAAGAACAGAUGCUGAGGCTT-3′,

siRNA2 sense,
5′-CCAGGAGAUUGUUGGAUAATT-3′,

and siRNA2 antisense,
5′-UUAUCCAACAAUCUCCUGGTT-3′.

3.3. Cell Culture and Transfection

The human cancer cells were grown in DMEM media (Sigma-Aldrich, USA) enriched with 10% inactivated fetal bovine serum (FBS, Gibco, USA), 100µg.mL^{-1} of each antibiotic (penicillin, and streptomycin) at 37 degrees Celsius and 5% CO_2. Transfection of cells (3×10^5 cells/transfection) was performed. PX459-sgRNA1 and PX459-sgRNA2 plasmids were co-transfected into cells mediated by

Lipofectamine™2000 (Invitrogen, USA) using the company's standard guidelines as a guide. As for the control group, the PX459- GFP vector was transfected into the cells of another well (PX459-group).

3.4. T7 Endonuclease I (T7EI)

In this test, after genomic DNA was extracted (DNPTM Kit, Tehran, Iran), cells were collected from cell culture 6- well plate and were centrifuged at 3000 rpm. The clean upper phase was scrapped and replaced with a 300 µL PBS higher part solutions. Cell Lysis was performed by resuspending induced cells into 25 µL Lysis Buffer. PCR was used to amplify nucleotide sequence comprising the target sequences of LINC00511 gRNAs. Then, 1 µL of T7 Endonuclease I kit (Genecopoecia TM, US) was added, incubated at a temperature of 20-60 °C. PCR products were run on a gel resolves full-length DNA, and PCR products cleavage was performed by T7 Endonuclease I enzyme.

3.5. Genes Amplification by Genomic PCR and Native PAGE

Cells were collected at 300×g for 5 minutes and washed in 0.45 % TWEEN 20, 0.45 % NP40, 0.1 $\mu g.mL^{-1}$ gelatine, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM TRIS pH 8.3 (cell lysis buffer). 20 $\mu g.mL^{-1}$ of proteinase-K (Cusabio, China) was added to the mixture. The cell lysates were then incubated for 1 hour at 55 degrees Celsius and 5 minutes at 95 degrees Celsius. DNA from the PCR reaction was denaturized and re-annealed at 0.1 C. s from 85 to 25 degrees Celsius for Native PAGE. The Purified DNA was then separated for 2 hours at 150 V on a 10% native Polyacrylamide gel. As a marker, the Genetic Ladder (Sina Yas, Iran) was utilized.

3.6. The Expression of Apoptosis Related Genes

The RNX™-Plus liquid was used to isolate RNA from the samples according with manufacturer guidelines (SinaClon, Iran). Additionally, utilizing random hexamer and the Prime Script™-RT kit (TaKaRa, Japan), 1µg of RNA was utilized to make cDNA. According to the manufacturer guidelines, quantitative RT-PCR was conducted using particular primers (**Table 1**) and the SYBR®Premix Ex Taq™ II kit (TaKaRa, Japan). The following conditions were used for thermal cycling: a 5-minute activation stage at 95 °C, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minutes. The normalizer was the GAPDH housekeeping gene.

Table 1. Sequence of primers used in this research

Gene	TM (°C)	Size (bp)	Primer sequence
SURVIVIN	64	170	F : 5′-AGAACTGGCCCTTGGAGG -3′ R : 5′-CTTTTTATGTTCCTCTATGGGGTC -3′
Bcl2	65	245	F : 5′-GACGACTTCTCCCGCCGCTAC -3′ R : 5′-CGGTTCAGGTACTCAGTCATCCAC -3′
BAX	65	234	F : 5′-AGGTCTTTTTCCGAGTGGCAGC -3′ R : 5′-GCGTCCCAAAGTAGGAGAGGAG -3′
GAPDH	64	183	F : 5′-GCCAAAAGGGTCATCATCTCTGC -3′ R : 5′-GGTCACGAGTCCTTCCACGATAC -3′
P53	64	170	F : 5′-TGCGTGTGGAGTATTTGGATGAC -3′ R : 5′- CAGTGTGATGATGGTGAGGATGG -3′
FAS	64	184	F : 5′-CAATTCTGCCATAAGCCCTGTC -3′ R : 5′- GTCCTTCATCACACAATCTACATCTTC -3′
MCF7, *NOX4*	63	517	F : 5′-GGGTCCAACACCACATAAATGTG-3′ R : 5′- GAGTCCGTATCTCCGAAACTCA-3′
HCA-7, *NOX4*	63	517	F : 5′-TGTGCCGAACACTCTTGGC-3′ R : 5′- ACATGCACGCCTGAGAAAATA -3′

The $2^{-\Delta\Delta Ct}$ approach was used to determine relative expressions.

3.7. Western Blotting

The cell lines were extracted and treated with a protease inhibitor mixture comprising 1 percent IPEGAL, 150mM NaCl, 0.5 percent deoxycholate, 5mM EDTA, 50mM Tris-HCl, pH 7.5 (RIPA buffer) (Promega, United States). Proteins including whole extracts were separated on SDS-PAGE and then applied to nitrocellulose sheets for protein immunoblot analysis. Odyssey was used to capture the photographs (Licor bioscience, Lincoln, NE). The *NOX1* (Abcam, United Kingdom) and *NOX4* (Sigma-Aldrich,USA), and GAPDH (WeiAo, China) antibodies were prepared.

3.8. Cell Proliferation, Cell Mortality, and Cell-Cycle Assay

In vitro proliferative activity was assessed with cancer cell lines using CyQUANT Cell Proliferation Assay Kit (C-7026). Freshly pelleted cells were cultured, and successive different concentration of cells in 200 mL were prepared on a microplate and cultured at 37 °C with 5% Carbon dioxide. Following 48 hours, each excellent growth media was changed with 100 mL of new media. Microplates were collected on days 0, 1, 2, 3, 4, and 9. At room temperature, all samples were dissolved, and 200 mL of CyQUANT GR dye/lysis solution (Life Technologies, Canada) was introduced to every unit plate. The mixture was incubated in dark environment for 5 minutes. The fluorescent of the samples was estimated, and proliferation graphs were calculated as fluorescent vs time. MTT (Sigma, MO, USA) assay measured cell mortality following the manufacturer's protocol. Propidium iodide (PI) was used to study cell divisions (BD Biosciences). Before flow cytometry, cell lines were rinsed in cool PBS, fixated in 75 percent ethanol at -20 °C 24 hrs, and then treated with PI for 15 minutes.

3.9. Flow-Cytometric Analysis of the Cell

Samples were evaluated using a flow cytometry technique with an Annexin V FITC dye (Thermo Fisher Scientific) to determine the amount of apoptosis, according to the company's procedure. The Results section used this apoptosis rate evaluation to analyze the correlation between apoptosis and gene expression levels obtained with real-time PCR.

3.10. Statistical Analysis

The average and standard deviation (SD) are used to show the data. The statistically significant difference of the discrepancies was determined using GraphPad Prism t-test. $P<0.05$ values were deemed significant in the analyses.

4. Results

4.1. Successful Knockout of NOX4 Using CRISPR/Cas9

The results of design, primer annealing location, and PX459-sgRNA 1 and PX459-sgRNA 2 vector containing cells PCR with specific primers are presented. According to the 289 and 287 bp bands in samples 2 and 4, the presence of sgRNA1 and 2 in vector PX459 are verified. Cell line cultures were monitored for 24-120hr post culture, and their growth profile was documented. Cell transfection to produce PX-459 was carried out by GFP containing cell lines free of sgRNAs and imaged after 24hr by fluorescence microscopy (**Fig. 1**). This picture shows a GFP vector containing cells as green dots. Considering the performance of PX459-sgRNA 1 and PX459-sgRNA 2 vectors in the cell lines, a 258bp segment of the *NOX4* must be deleted. Therefore, the expected PCR product for target groups (PX459-*NOX4*-sgRNA1,2) is 550bp, and 808bp for the control (PX459 and blank) (**Fig. 2**). The existence of a 550bp band in the PCR products shows the valid function of the sgRNA. To further assess if the CRISPR sgRNAs have only targeted the intended *NOX4*I, the T7 endonuclease I was used for cutting the target genes. The 550bp band was extracted for this purpose and treated with T7 endonuclease I, which split the band into 185 and 365bp products. According to results, the two 185 and 365 pieces represent the valid functioning of the sgRNAs. Co-transfection of the recombinant constructs to the MCF-7, UM-RC-6, and HCA-7 cells resulted in 550bp bands after PCR analyses using *NOX4*-F and *NOX4*-R primers. The same PCR analyses resulted in an 808 bp and the other two groups of cells (blank control group and vehicle group PX459-GFP), indicating no gene editing. Since the deleted fragment includes parts of the promoter region and exon 1 of the *NOX4* gene, this gene's CRISPR/Cas9 mediated knockout appears to be entirely successful. DNA repair process in crisper

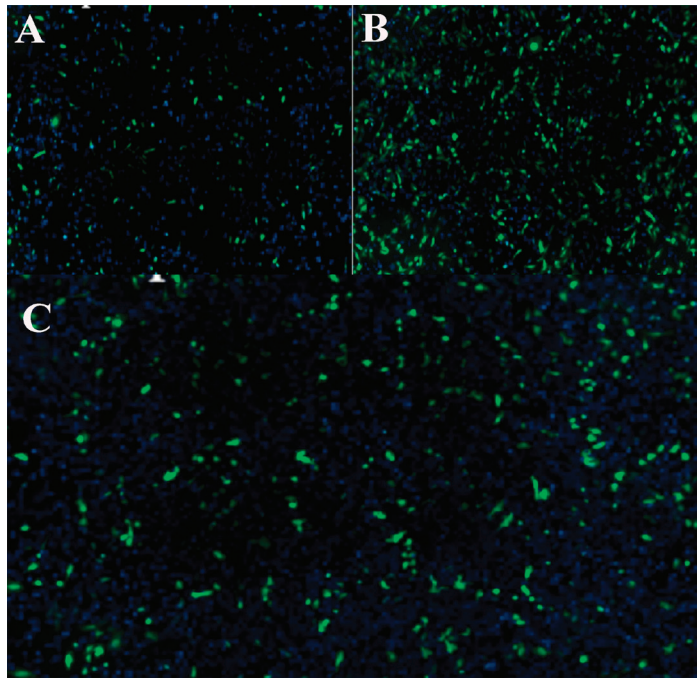

Figure 1. Transfection of cells: **A)** MCF7, **B)** UM-RC-6, and **C)** HCA-7 using the Lipofectamine 2000 reagent. According to the transfection results, cell line UM-RC-6 had a higher GFP transfection efficiency than MCF-7 and HCA-7.

technique is mainly performed by Homologous End Joining (NHEJ). This process is prone to error, so that some indels may cause slightly different fragments. Based on theory evidence, the expected length of the fragment is 365bp. A T7 endonuclease 1 assay was used to confirm the CRISPR system efficiency. **Figure 2** shows the results of the cleavage assay using the T7 endonuclease. The presence of cleaved mismatch bands confirms *NOX4* knockout (**Fig. 2**, Lane 4, 5, 7). All three MCF-7, HCA-7, and UM-RC-6 cell lines have Cleaved mismatch products segment, and bands of 185, 365, and 550 bp indicate correct knockout confirmation. It is in all three cell lines.

4.2. CRISPR/Cas9-Mediated NOX4 Knockout Validated at mRNA and Protein Levels

The knockout experiments were performed on a cell pool of each cell line. So, a section of the cell population was expected to be targeted by the application CRISPR knockout technique. Therefore, the treated cell pool must be heterogeneous. So, the treated cell population tested was heterogeneous in terms of silencing the gene activity. This heterogeneity was observed in experiments performed on genomic DNA and Western blotting. According to the result, UM-RC-6 kidney cancer cell lines had the highest level of mRNA expression, followed by HCA-7 colorectal cancer cell lines and the MCF7 cell line. In all three cell lines, after knocking out *NOX4* (PX459-sgRNA1,2), mRNA expression was decreased, which shows the proper function of the CRISPR system and a significant difference (**P<0.001) with the control cell lines. According to the results, the greatest effect of crisper on cell mass was HCA-7, followed by MCF-7 and UM-RC-6, respectively. Therefore, the lowest *NOX4* gene knockout was seen in UM-RC-6 kidney cancer cell lines, which was visible at a significant level of $P < 0.05$ compared with the control group (**Fig. 2B**).

Furthermore, the results of western blots using NOX4 antibodies revealed that the protein's expression is lost in *NOX4* knockout cells (target groups) (**Fig. 2C**), while *NOX4* had a strong expression in the control group. On the other hand, *NOX1* expression in the target group (*NOX4* knockout) was used as an internal control. The results demonstrate that *NOX1* was expressed in these cells (**Fig. 2C**). These results were confirmed by western blotting, which revealed that the protein band of the UM-RC-6 cell group was stronger than that of the

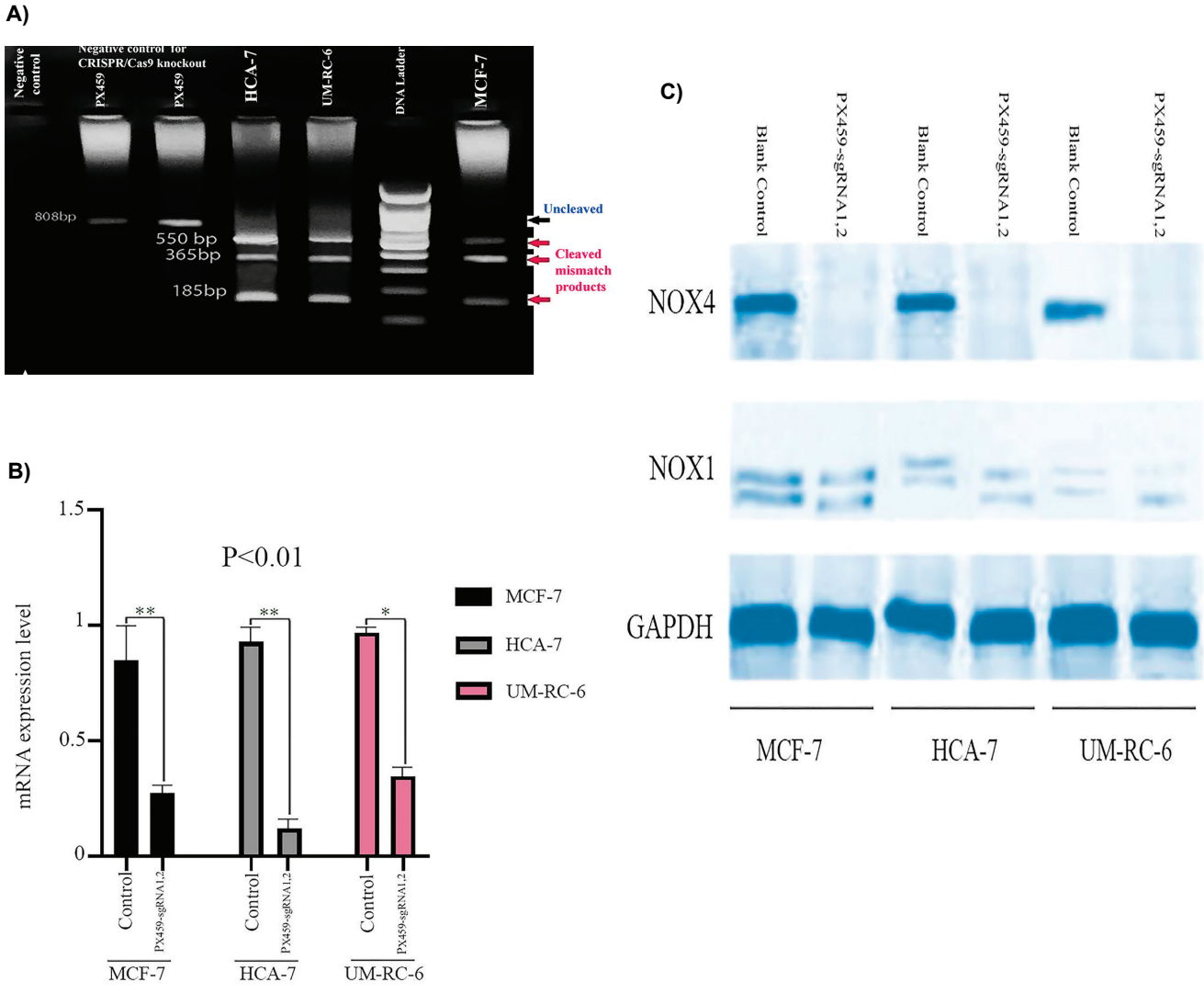

Figure 2. A) PCR analysis of gene expression in MCF7, UM-RC-6 and HCA-7 transfected cells. Lane1: Negative control for the PCR reaction; Lane 2 and 3: PX459-groups (800 bp Band showing in Negative control group for CRISPR/Cas9 knockout); Lane 4: CRISPR/Cas9 knocked out HCA-7 DNA digested by T7E1 (185, 365, 550 bp Bands after T7 endonuclease function have been seen); Lane5: CRISPR/Cas9 knocked out UM-RC-6 DNA digested by T7E1 (185, 365, 550 bp Bands after T7 endonuclease function have been seen); Lane 6: 100bp DNA Ladder; Lane 7: CRISPR/Cas9 knocked out MCF-7DNA digested by T7E1 (185, 365, 550 bp Bands after T7 endonuclease function have been seen). **B) *NOX4* expression in MCF-7, UM-RC-6, and HCA-7 cells compared to control groups.** after knocking out *NOX4* (PX459-sgRNA1,2) mRNA expression was decreased which shows the proper function of the CRISPR system and a significant difference (**P<0.001) with the control cell lines. According to the results, the greatest effect of crisper on cell mass was HCA-7, followed by MCF-7 and UM-RC-6, respectively. **C) Western blot results (NOX4 protein expression) with anti-NOX4 and NOX1 antibodies.** Equal amount of control and NOX4 knockout cell lysates were probed with anti-*NOX4* antibody. Experiments were repeated three times with similar observations, and representative data is shown. *NOX4* knockout did not influence NOX1 levels. Protein levels of NOX1, in parental cells and three clones of *NOX4* knockout. The knockout experiments were performed on a cell pool of each cell line. So, it is expected that a section of the cell population, was targeted by the application CRISPR knockout technique. Therefore, the treated cell pool must be heterogeneous. So, the treated cell population tested was heterogeneous in terms of silencing the gene activity. This heterogeneity was observed in experiments performed on Western blotting.

MCF-7 and HCA-7 cell lines. These results show that the CRISPR-Cas9 mediated *NOX4* knockouts are valid in the target cells at a cellular level (gene and protein).

4.3. Effect of NOX4 Knockout on the Expression of Apoptosis-Related Genes

In the *NOX4* knockout, we expect to have an increase in apoptotic gene expression and a lowered expression of anti-apoptotic gene expression. According to the results (**Fig. 3**), the anti-apoptosis genes *BCL2* and *SURVIVIN* were over-expressed in control compared to the target (PX459-sgRNA1,2) in the three cell lines, while the expression of the apoptotic genes *P53*, *BAX*, and *FAS* were increased in the three mentioned cell lines of *NOX4* knockouts (target group) compared to the blank control group. The P-value for the pro/anti-apoptosis genes was significant according to our calculations. In contrast, the *BCL2* and *SURVIVIN* genes in target groups demonstrated loss of *NOX4*. These results show that by removing *NOX4* in cancer cells, the apoptosis genes increase expression and the anti-apoptosis genes have lowered expression. Additionally, *NOX4* knockouts increase apoptotic gene expression in breast, colorectal, and kidney cancer. These results demonstrate the importance of *NOX4* in the mentioned cancers.

4.4. CRISPR/Cas9-Mediated Knockout of NOX4 Decreased Cell Proliferation

The effect of *NOX4* knockout on cancer cell lines MCF7, UM-RC-6, and HCA-7 were evaluated in 24, 48, 72, 96, 120, 144, 168hr. According to results (**Fig. 3**), cancer cell proliferation of MCF7, UM-RC-6, and HCA-7 lines were significantly lowered in

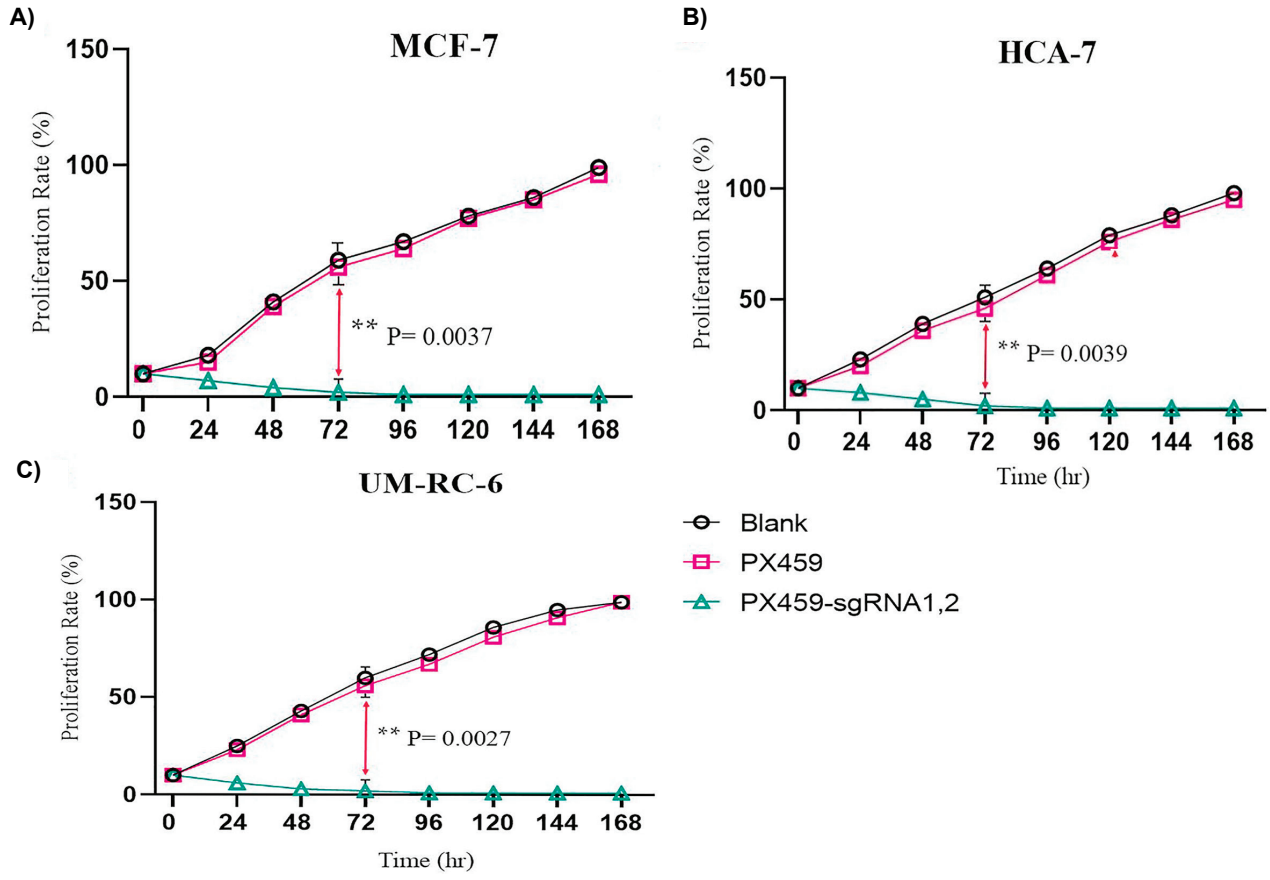

Figure 3. The effect of NOX4 knockouts on MCF-7, UM-RC-6, and HCA-7 cell lines. The cancerous cell proliferation rate is lowered significantly after 72hr in the PX459-sgRNA1, 2 group compared to the blank control and PX459.

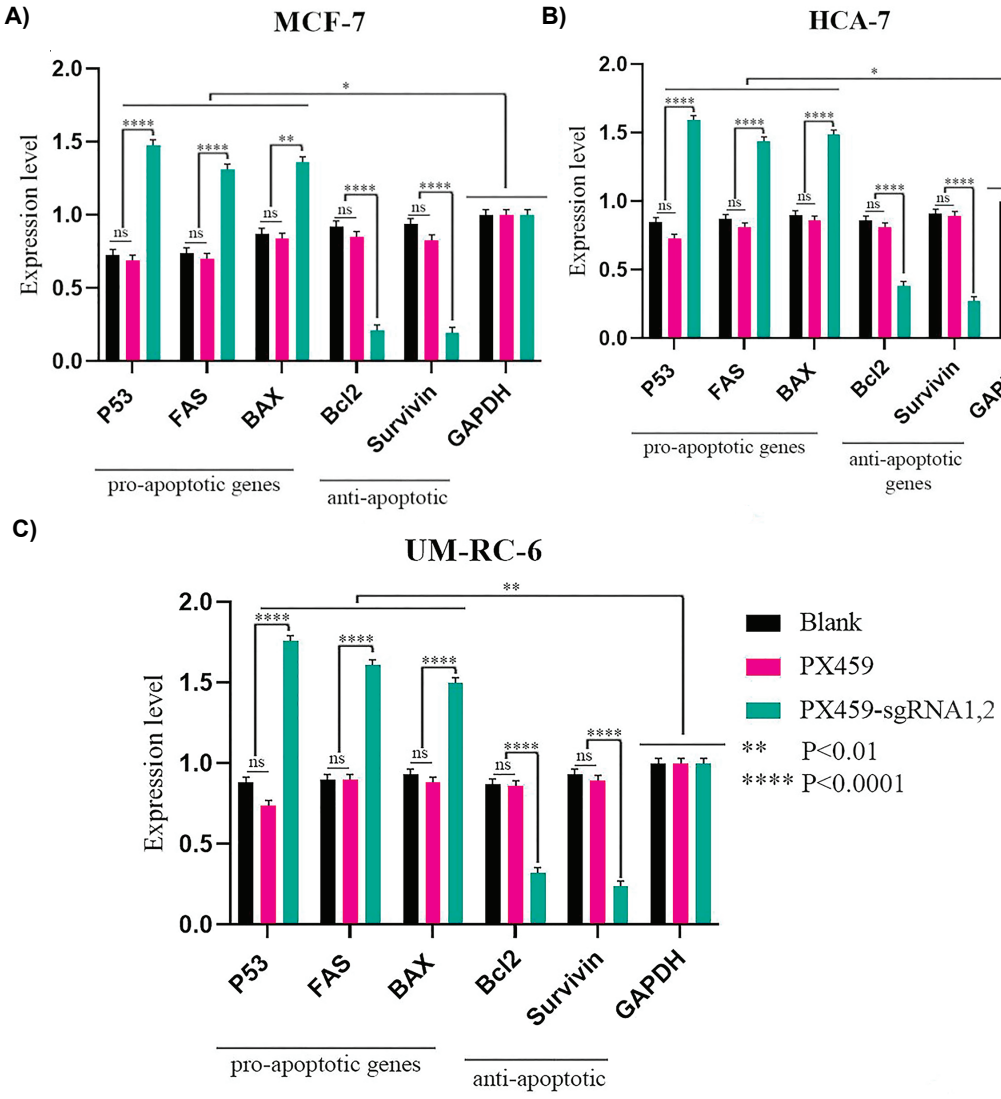

Figure 4. Changes in the balance of pro- and anti-apoptotic gene expression. The sum of the expression levels of anti-apoptotic genes; (*SURVIVIN* and *BCL2*) and pro-apoptotic genes (*BAX, P53* and *FAS*) in: **A)** breast (MCF7), **B)** colon (HCA-7), and **C)** Kidney (UM-RC-6) cancer cell lines were compared by Real-Time PCR. As a result, expression of the anti-apoptotic factors *BCL-2* and *SURVIVIN* were decreased, whereas expression of the pro-apoptotic factors *BAX, FAS* and *P53* increased.

72hr in the PX459-sgRNA1,2 group compared to the control (PX459) and blank control group (P<0.001). These results show lowered cell proliferation caused by a *NOX4* knockout and correct functioning of the CRISPR system. The effect of *NOX4* knockout on mortality rates of cell lines MCF7, UM-RC-6, and HCA-7 in 24, 48, 72hr has been assessed by the MTT method. The MTT assay results show a significant (P<0.01) difference between the growth of the control group (blank and PX495) and the target group (PX495-sgRNA1,2) in all cell lines. In 72hr, the mortality rate of the cancer cell lines MCF7, HCA-7, and UM-RC-6 are 59%, 61%, 68%. These results are by the real-time PCR and show the effects of NOX4 knockout on the MCF7, UM-RC-6, and HCA-7 cell lines (**Fig. 4**).

Subsequently, the cell cycle was assessed with flow cytometry. In the target group, cell lines (pX459-sgRNA1,2 group), sub-G1 was increased compared to pX459 and the control blank, which caused delayed entry to the G2 phase and a subsequently reduced

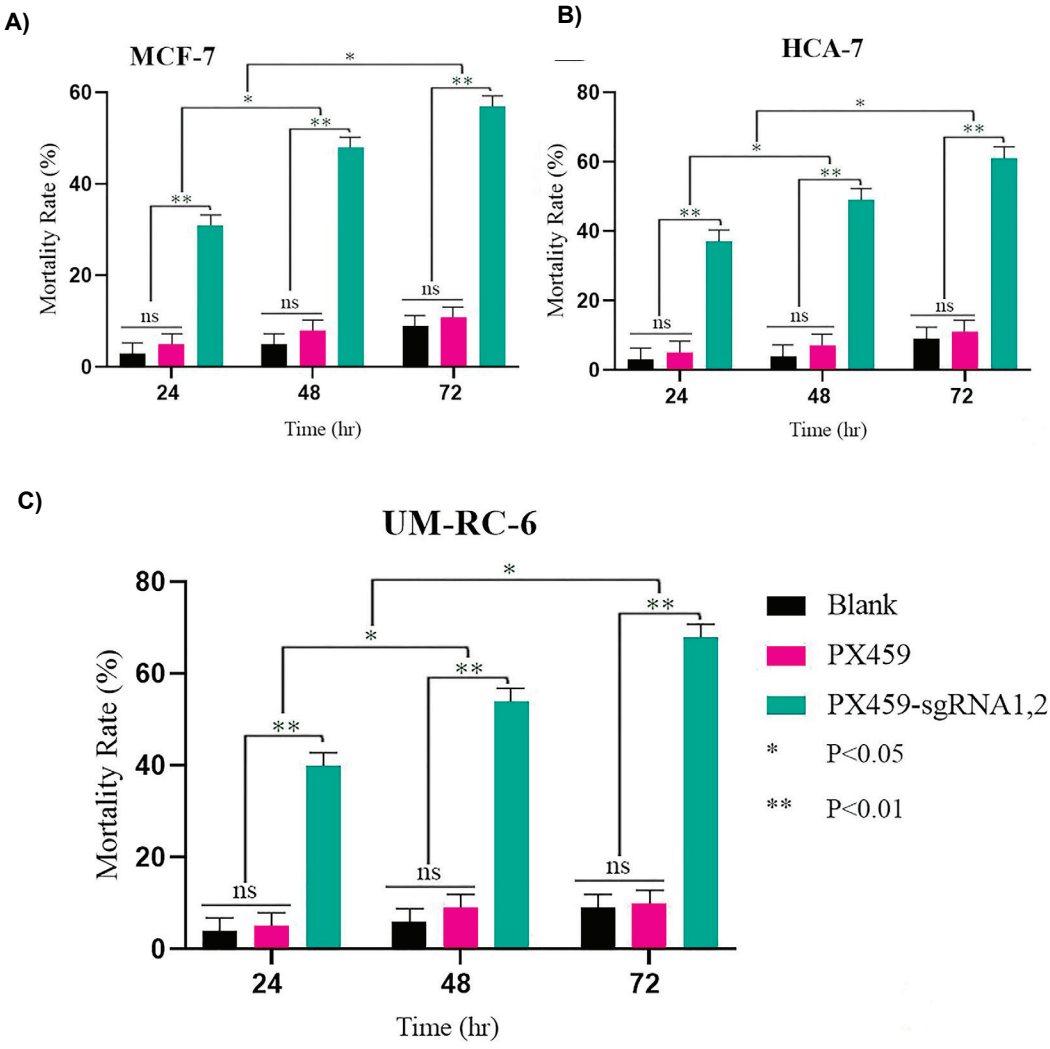

Figure 5. MTT assay. Cell mortality in cell transfected (PX459-sgRNA1, 2) **A)** MCF7, **B)** HCA-7, and **C)** UM-RC-6, cell lines were significantly difference with compared to control cells. The differences at 24, 48 and 72 hours were considered significant when p value was lower than 0.05. Adjusted P Value Blank and pX459 Control groups vs pX459-sgRNA1,2 <0.01.

cell proliferation rate in this group. The DNA amount of the cells was determined using flow cytometry after they were treated and labeled with propidium iodide. The outcome of one typical test from three different experiments is displayed. The x and y axes represent the amount of DNA and the number of cells, correspondingly. The cell-flowing program was used to compute each cycle. Sub-G1, G0/G1, S, and G2/M cell percentages were also shown. The data is presented as a mean with standard deviation (SEM) (mean ± SEM). P<0.0001 (one-way analysis of variance followed by independent sample t-test). Three times, the experiment was conducted.

4.5. Apoptosis Rate Increase

In this study, the V-FITC Annexin dye was used to assess the primary apoptosis, secondary apoptosis, necrosis, and living cells in three cell groups (blank control, PX459, and the PX459-sgRNA1,2 group). According to the results (**Fig. 5**), the PX459-sgRNA1,2 group (cells with a knocked out *NOX4*) of MCF7 had primary apoptosis, secondary apoptosis, necrosis, and living cell rate of 28.25%, 32.87%, 9.89%, and 28.99%, and the HCA-7 had rates of 23,97%, 31.61%, 10.2%, and 34.22% while the UM-RC-6 kidney cancer cell line had more apoptosis with rates of 31.25%, 37.87%, 4.49%, 26.39%. The control cells had primary

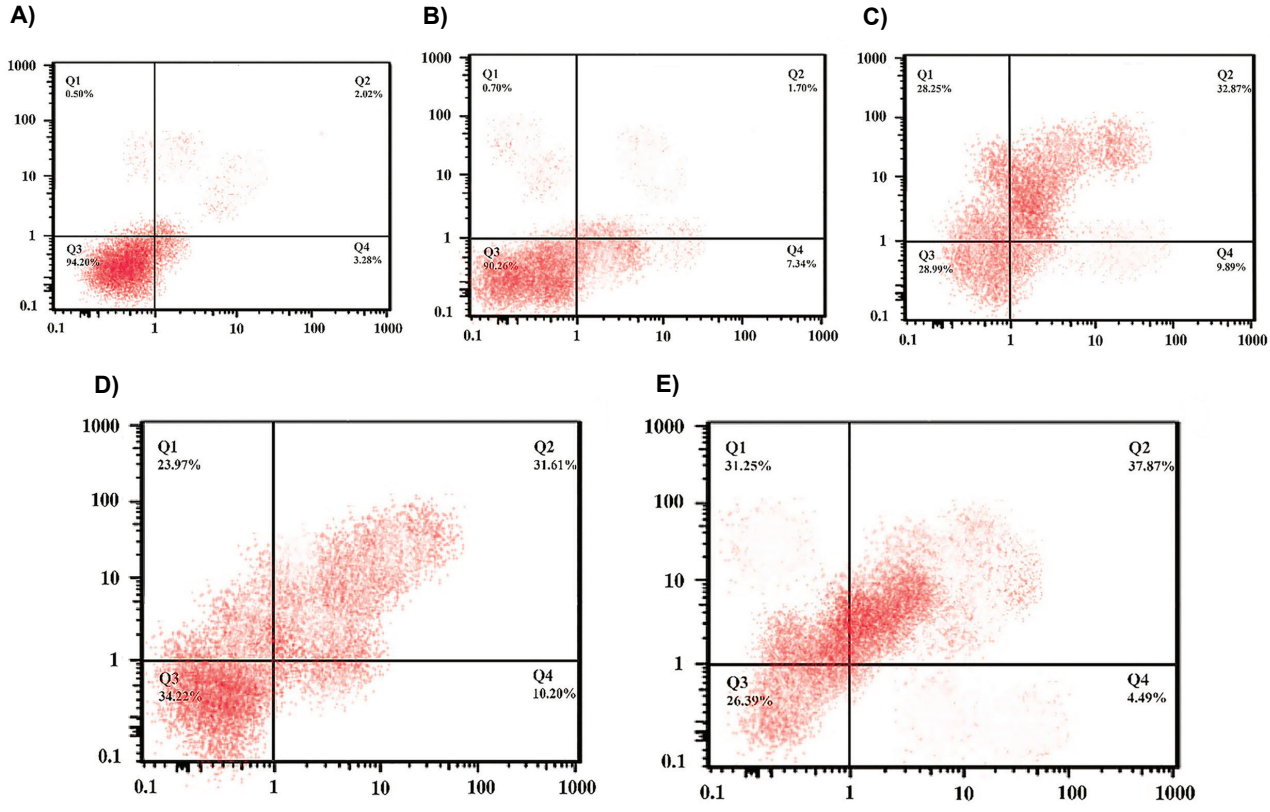

Figure 6. The amount of primary apoptosis, secondary apoptosis, necrosis, and living cells in three groups of cells based on coloring with the annexin V-FITC dye. **A)** Blank control group, **B)** PX459 group, **C)** PX459-sgRNA1,2 group of MCF-7 cell line, **D)** PX459-sgRNA1,2 group of HCA-7 cell line, and **E)** PX459-sgRNA1,2 group of the UM-RC-6 cell line.

apoptosis, secondary apoptosis, necrosis, and living cell rates of 0.5%, 2.02%, 7.22%, 90.26%. The results above confirm the MTT and real-time PCR results that the knockout of *NOX4* causes apoptosis in cancer cells MCF7, UM-RC-6, HCA-7 (**Fig. 6**).

5. Discussion

The current study shows successful *NOX4* knockouts by the CRISPR/Cas9 system in breast cancer (MCF7 cell lines), kidney cancer (UM-RC-6), and colorectal cancer (HCA-7) cell lines (18, 19, 20). In this study, a growth graph was drawn after growing MCF7, UM-RC-6, and HCA-7 lines. The UM-RC-6 cell line had the largest growth rate, and HCA-7 had the lowest growth rate. This result agrees with Zhang *et al*. (2009) finding, showing that kidney cancer cell lines grow FAS more than other cell lines (21). The NOX4 is a membrane-associated active redox protein that unstably plays a role in human cancers through irradiation sensitivity, oxidation, and signaling based on H_2O_2 production capacity (22). The catalytic component of the NADPH oxidase compound is encoded by this gene, which belongs to the NOX family of enzymes. Non-phagocytic cells have the encoding polypeptide, which functions as an oxygen sensor and stimulates the reduction of oxygen molecules to different reactive oxygen species (ROS). This protein's ROS has been linked to a variety of biological processes, involving signal transmission, cell differentiation, and malignant cells proliferation (22). *NOX4* overexpression has been seen in multiple tumor types (23). This report used the CRISPR-Cas9 gene-editing system to knock out *NOX4* in cancer cell lines MCF7, UM-RC-6, and HCA-7. Most of the *NOX4* gene knockout effect is related to colon cancer and breast cancer cell and renal cancers. These results were confirmed by western blotting, which revealed that the protein band of the UM-RC-6 cell

group was stronger than that of the MCF-7 and HCA-7 cell lines. These results show that the CRISPR-Cas9 mediated *NOX4* knockouts are valid in the target cells at a cellular level (gene and protein). As far as we know, this is the first study focused on the comparative assessment of *NOX4* knockouts of three cancers. In agreement with Jafari *et al*. (2017), this study found that mRNA expression of *NOX4* is lowered considerably in target cells, leading to a lowered *NOX4* protein level (26). In this study, we observed a significant lowering of MCF7, UM-RC-6, and HCA-7 cancer cell line›s proliferation after deleting *NOX4* compared to the control group. Because of *NOX4*'s involvement in multiple cellular processes, i.e., regulation of apoptosis regulators and cell cycle growth factors inhibits apoptosis and promotes cancer cell proliferation (24).

Interestingly, *NOX4* isoforms are rarely directly associated with cancer spreading (25). The involvement of NOX4 in various malignancies has been the subject of several studies. The effect of NOX4 in cancer progression is unknown due to the contradictions in these results; although several research show NOX4 assists cell growth, others claim NOX4 suppresses cell growth and aids liver cell apoptosis (27, 28, 29). Additionally, *NOX4* knockouts of the three MCF7, UM-RC-6, and HCA-7 lines cause lowered expression of the anti-apoptosis genes *SURVIVIN* and *BCL-2* followed by a significant ($P<0.0001$) expression increase in the *BAX*, *P53*, *FAS* genes compared to the control group. In-vitro silencing of *NOX4* in cells caused lowered expression in *BCL-2* and *SURVIVIN,* which caused an increased tendency of apoptosis in cancer cells. This result agrees with the study of Nlandu-Khodo et al. (2016), showing that the overexpression of an active form in the *NOX4* knockouts causes the death of cancer cells (30).

Additionally, the current study's findings reveal *NOX4* is needed for the proliferation of MCF-7, UM-RC-6, HCA-7 cell lines (31, 32, 33). Cell cycle analysis of *NOX4* knockouts of cancer cell lines showed the population of M/S/G2 cells was lowered (34, 35, 36). According to these findings, removing the *NOX4* gene inhibits cell proliferation. This study also confirms the studies, which showed that creating *NOX4* knockouts, the prophase of mitosis is expanded, which delays entry to the G2 stage of the cell cycle and finally results in cell death (37, 38). As these findings grow, we suggest further study of the mechanisms causing cell proliferation in cancers by *NOX4* and feedback from other NOX isoforms that affect *NOX4* activation. Additionally, to our knowledge, this is the first study on the effect of *NOX4* on the progression of cancer cells. Furthermore, using *NOX4* as a target can have therapeutic value for creating potential treatments against breast, colorectal, and kidney cancers which shows a need for a deeper understanding of the biology of these cancers with direct clinical outcomes for developing novel treatment strategies.

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