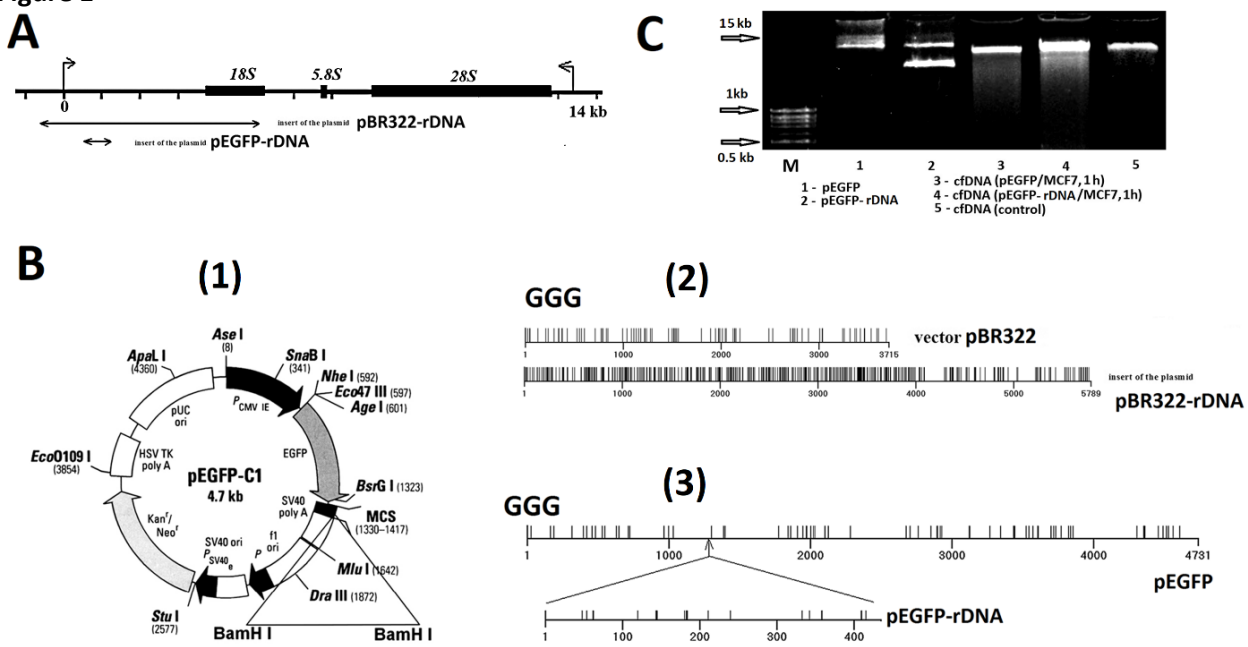
Figures with comments on «***The extracellular DNA containing easily oxidizable Gc-Rich motifs of human ribosomal DNA stimulates ROS generation in MCF7 cancer cell, is oxidized, penetrates the cell and can be expressed***» researching

Figure 1



A. *Scheme of the human ribosomal repeat. Segments – the inserts of the model GC-DNA analyzed are shown.* ***B.*** *(**1**) - Plasmid pEGFP-C1 (pEGFP) (http://www.bdbiosciences.com, GenBank accession number U55763). The site BamH1 is marked. (**2**) and (**3**) - Distribution of the easily oxidizable GGG - motifs within the pBR322, pBR322-rDNA, pEGFP and pEGFP-rDNA.* ***C.*** *Determination of the plasmid fragmentation in the extracellular DNA after 1 h of MCF7 incubation with 50 ng/mL pEGFP or pEGFP-rDNA. The total DNA was isolated from the cell culture medium. Electrophoresis of DNA was carried out in an 2 % agarose gel stained with ethidium bromide. Gel tracks 1 and 2 - plasmid solutions in water were applied.*

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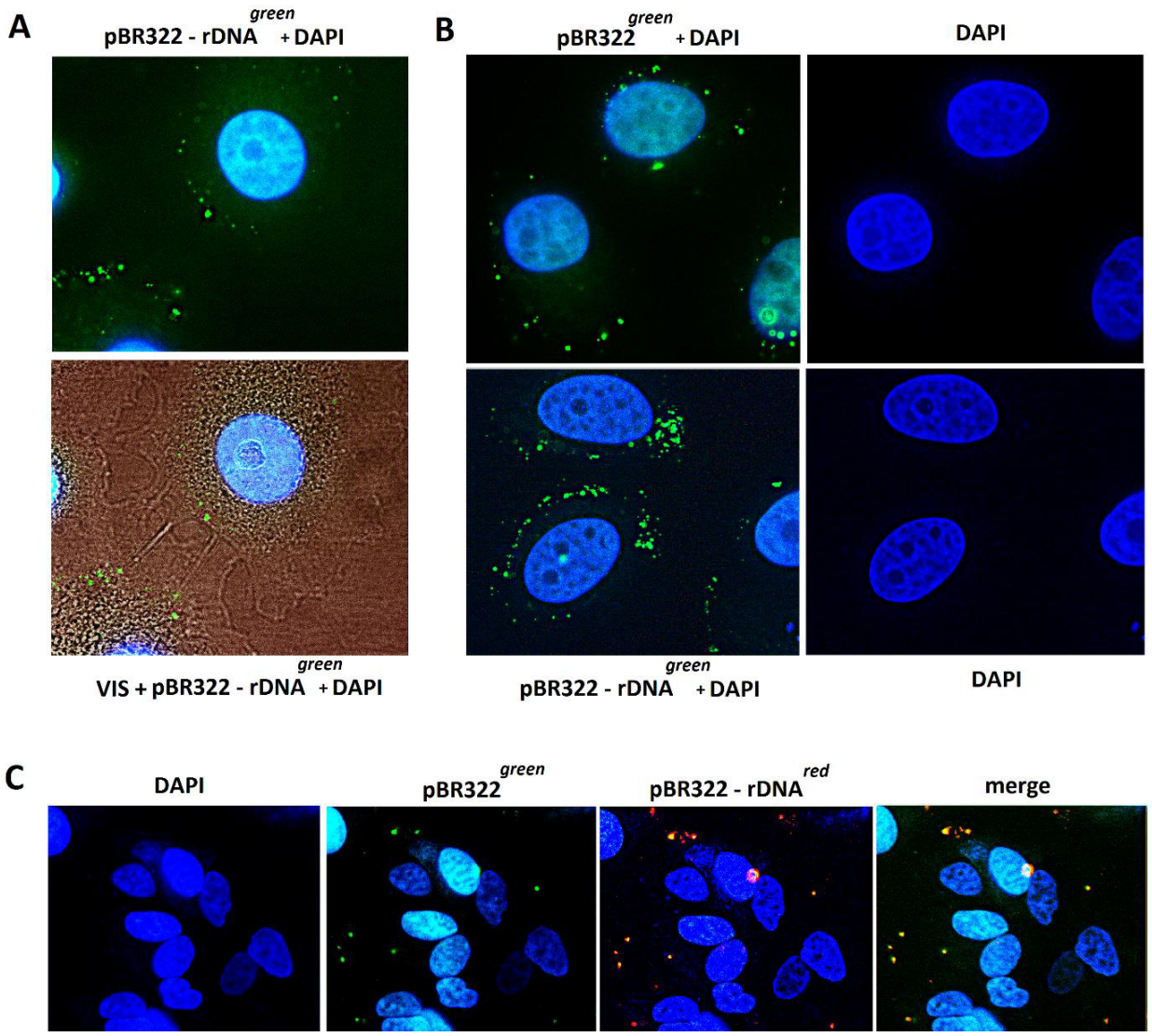
Figure 2. Staining of MCF7 cells with various types of labeled GC-DNA



A. *Top photo - pBR322 – rDNA* ^{green}*, nuclei are stained with DAPI. Bottom photo - merged staining patterns of pBR322 – rDNA* ^{green}*, DAPI and the image of the cell in visible light, (x100).* ***B.*** *pBR322* ^{green} *(top photo) and pBR322 – rDNA* ^{green} *(bottom photo).* ***C.*** *Merged staining patterns of pBR322* ^{green} *and pBR322–rDNA* ^{red} *(x40). Cells were incubated with GC-DNA for 30 min before fixation in 3% formaldehyde.*

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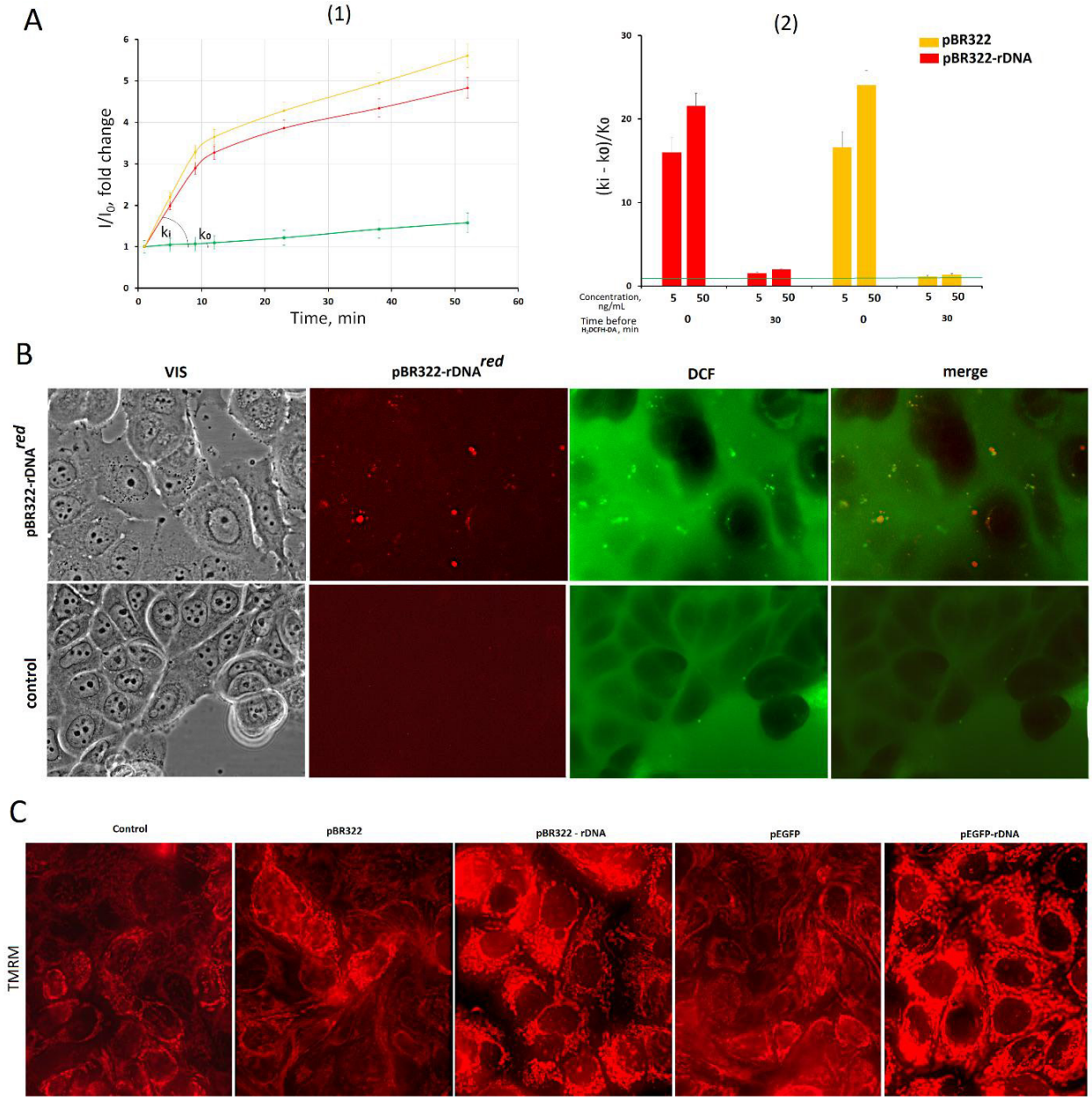
Figure 3. The exposure of MCF7 cells to GC-DNA leads to an increase in ROS production



A. *FL-plate reader. (**1**) - Example of reaction rate constant determination for DCF formation. The cultivation environment was replaced by 5 µm H2DCFH-DA in PBS solution and a relative fluorescence intensity increase was detected. I, I_0 – sample's signal at time t and immediately after H_2DCFH-DA addition, respectively. The line slope – reaction rate constant for DCF formation (k). ROS index $\Delta k/k_0 = (ki-k_0)/k_0$. (**2**) - ROS index. Time of cultivation with GC-DNA before adding H2DCFH-DA is shown in the figure.* ***B.*** *FM -based evaluation of MCF7 cells sequentially treated with 5µm H_2DCFH-DA and pBR322–rDNA^{red} (50 ng/mL) and incubated for 30 min (x40).* ***C.*** *MCF7 cells exposed to GC-DNA (30 min; 50 ng/mL), were treated with Mito-tracker TMRM (15 min).*

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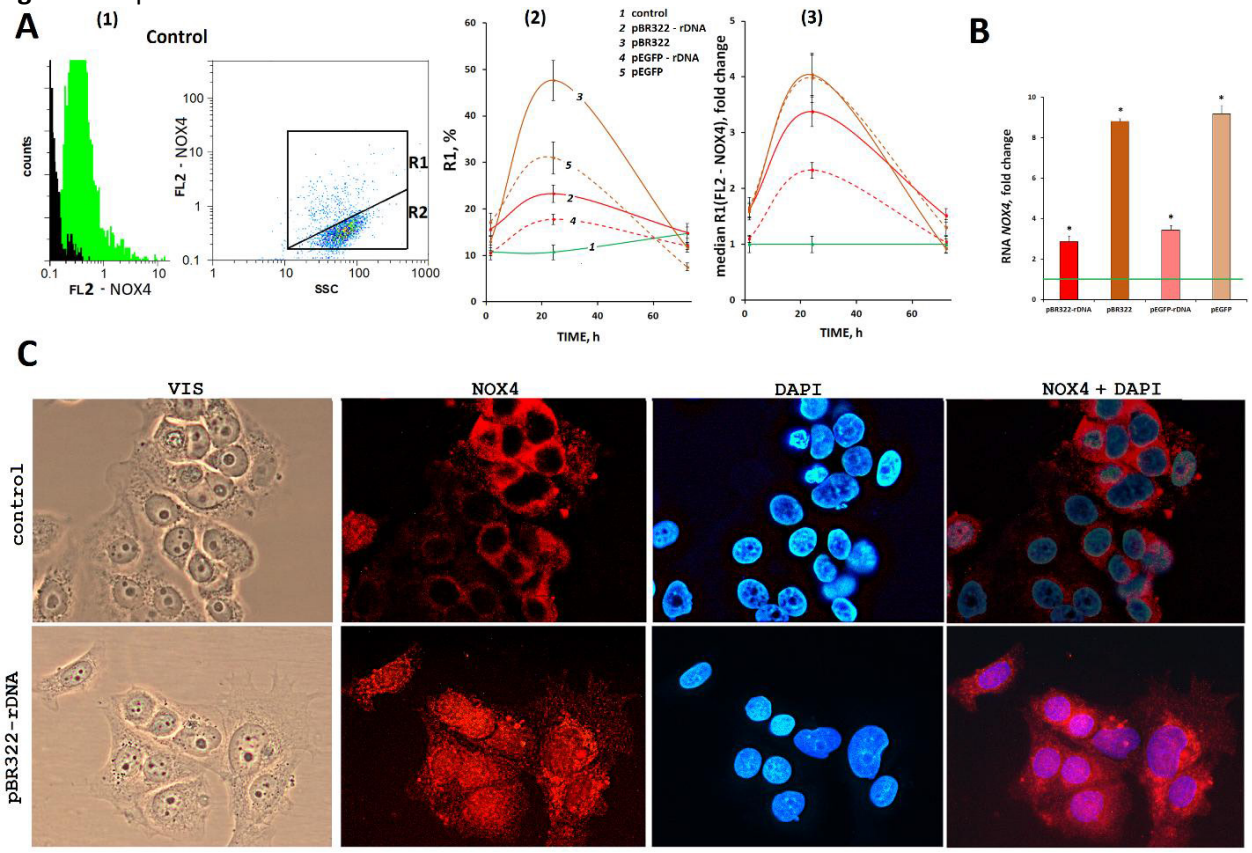
Figure 4. Expression of NOX4 in MCF7



A.*FCA. (**1**) - The distribution of FL2- NOX4 fluorescence intensities; black - background fluorescence (PE conjugated secondary antibodies). Cells plots: FL2 (NOX4-PE) versus SSC, R1 and R2: gated areas. (**2**) - relative fractions of NOX4-positive cells in R1 gate. (**3**) - median signal intensity of FL2- NOX4 in R1 gate.* ***B.*** *RT-qPCR. The levels of NOX4 – encoding RNAs in the cells exposed to GC-DNA for 24 h. The data are normalized by the content of RNA NOX4 in the control.* ***C.*** *FM-based evaluation of NOX4 (PE) in the cells treated with pEGFP-rDNA for 24 h (X40).* *$p < 0.05$ against control group of cells, non-parametric U-test.*

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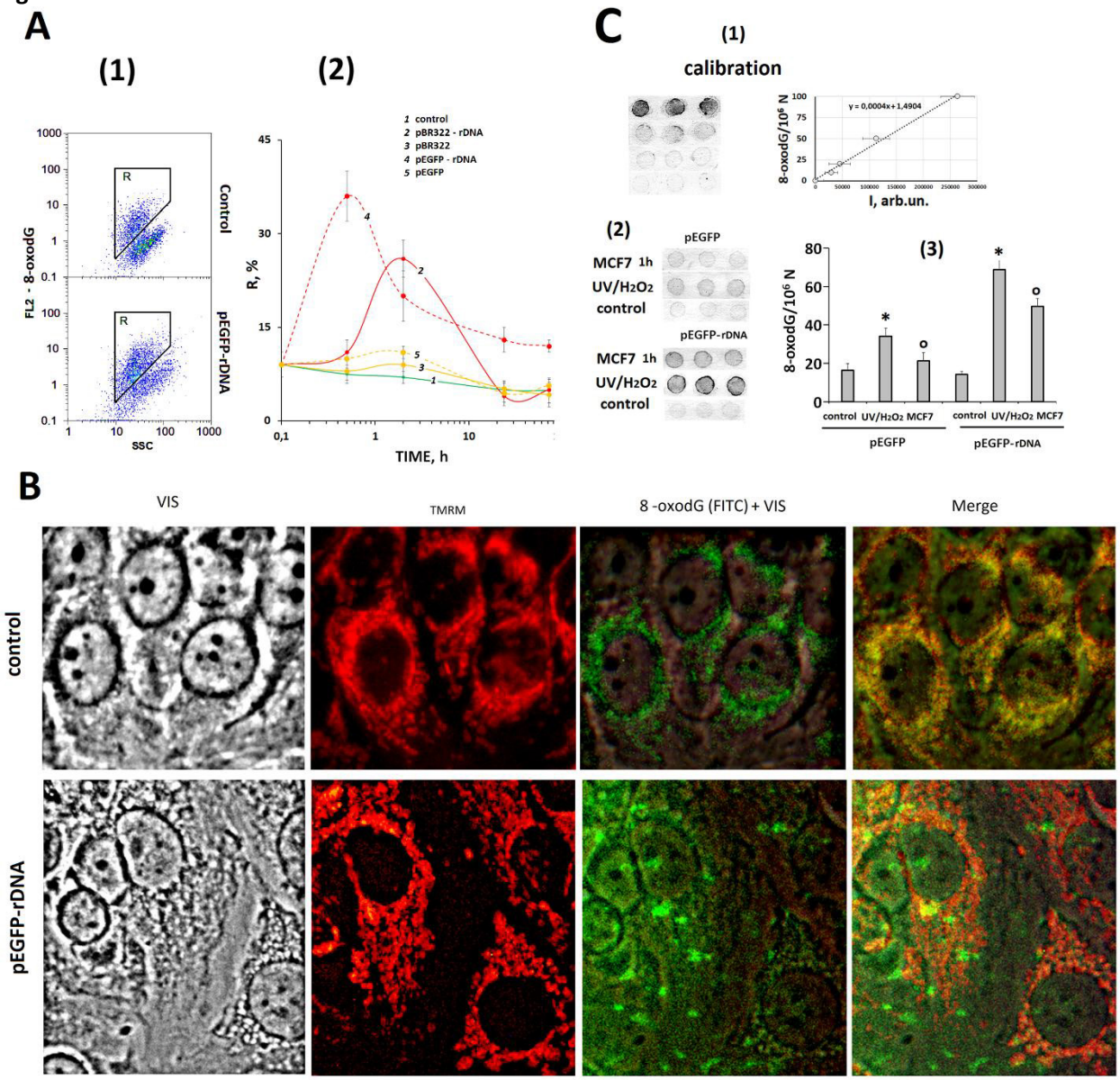
Figure 5. 8-oxodG levels in MCF7



A.*FCA. (**1**) - Cells plots: FL2 (8-oxodG-PE) versus SSC. R: gated area. (**2**) - Relative proportions of 8- oxodG positive cells in R gate (change with time). **B.** FM-based evaluation of mitochondria and 8-oxodG (FITC) in the cells treated with pEGFP-rDNA for 1 h (X40). **C.** 8-oxodG levels in pEGFP and pEGFP-rDNA. Immunoassay technique on nitrocellulose membranes using 8-oxodG antibodies conjugated with alkaline phosphatase was used. (**1**) and (**2**) - Four standard samples of oxidized genomic DNA (10 ng/dot) with a known content of 8-oxodG were applied in order to plot a calibration curve for the dependence of the signal intensity on the number of 8-oxodG copies. (**3**) and (**4**) - The samples of the oxidized and non-oxidized (control) pEGFP and pEGFP-rDNA (10 ng/dot) were applied**.** MCF7 1 h **- pEGFP or pEGFP-rDNA** after 1 h of incubation with MCF7; UV/H2O2 - plasmids were oxidized in 0.1% H_2O_2 solution with UV irradiation (λ> 312 nm).*

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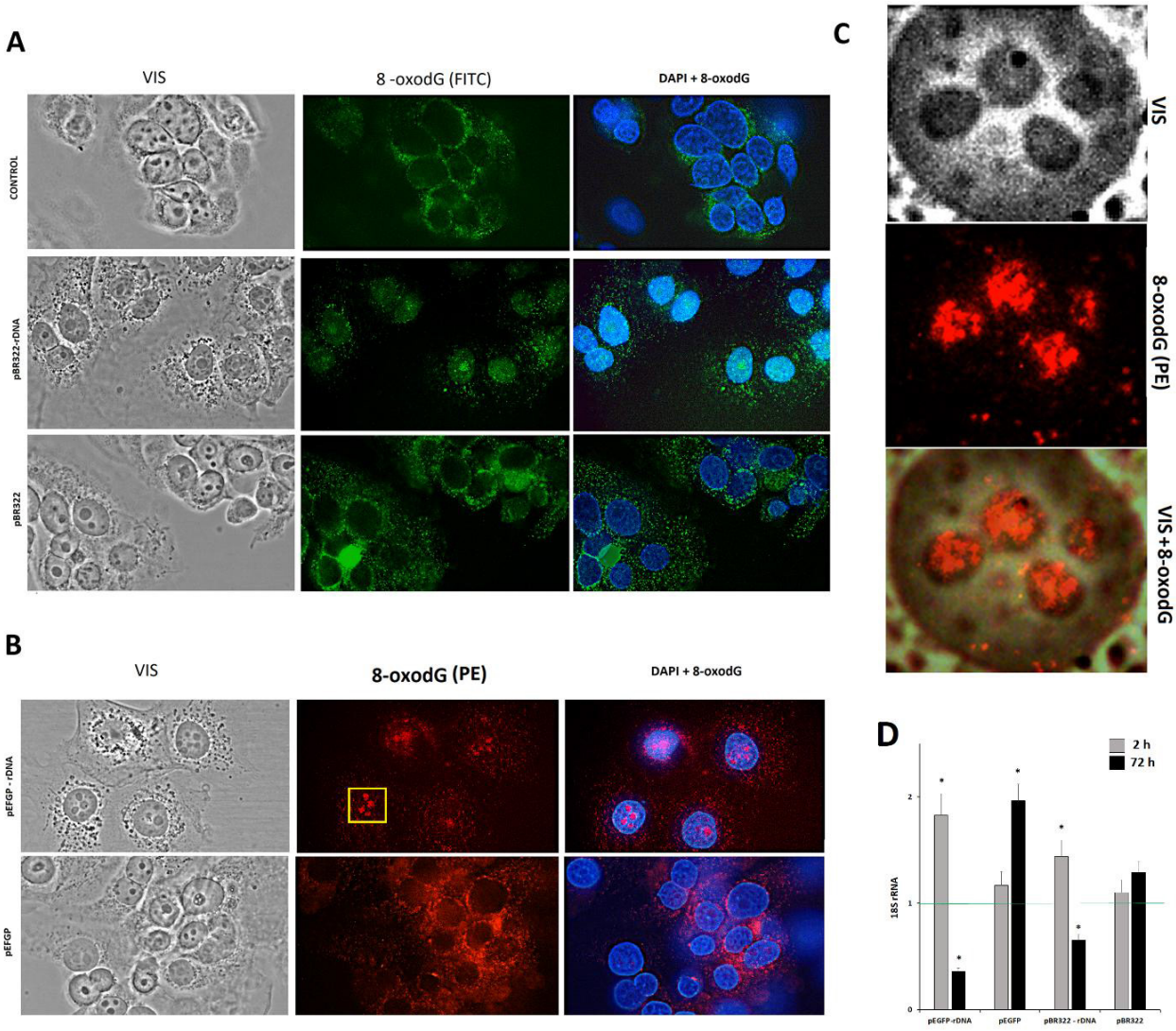
Figure 6



*FM-based evaluation of 8-oxodG in the cells treated with **pBR322, pBR322-rDNA (A) and pEGFP, pEGFP-rDNA (B)** for 24 h (X40). **C.** For the analysis of the selected area of the photo (B) the image size was increased by computer processing. **D.** RT-qPCR. The levels of 18S rRNA in the cells exposed to GC-DNA for 2 h and 24 h. The data are normalized by the content of 18Sr RNA in the control.*

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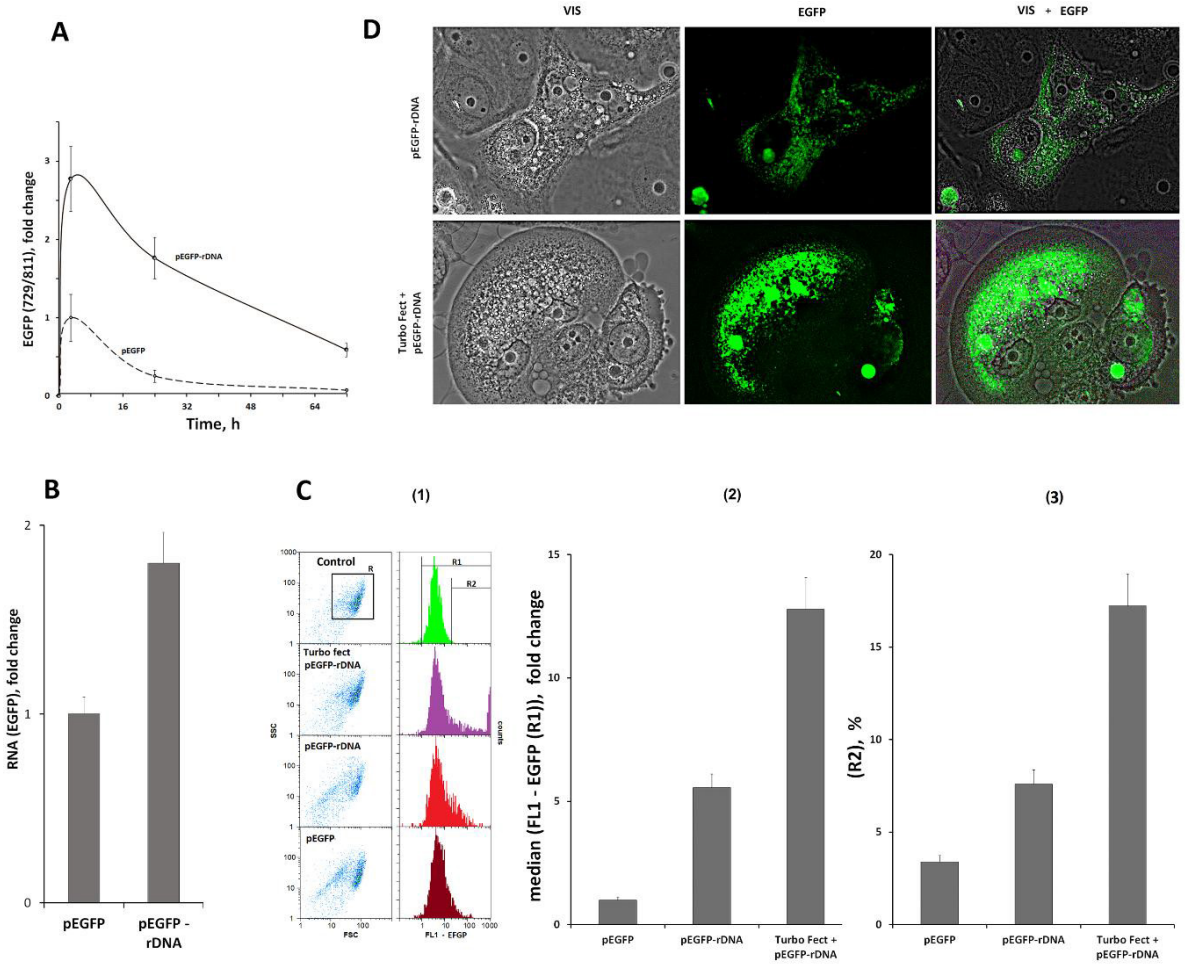
Figure 7. Expression of EGFP in MCF7 treated with pEGFP-rDNA or pEGFP for 24 h



A. *qPCR. Quantitation of* ***pEGFP and pEGFP-rDNA in the cellular DNA*** *after 3, 24, and 72 hours of incubation;* ***pEGFP*** *content (3 h) is taken as one unit.* ***B.*** *RT-qPCR. The levels of EGFP – encoding RNAs in the cells exposed to pEGFP or pEGFP-rDNA.* ***C.*** *FCA. (**1**) – non fixed cells plots: SSC versus FCS; R- the analyzed cell subpopulation. Distribution of FL1-EGFP fluorescence intensities. R1 and R2 - gated areas. (**2**) - Median signal intensity of of FL1- EGFP (gate R1). (**3**) - Relative fractions of EGFP-positive cells (R2 gate). D. FM-based evaluation of EGFP in the cells treated with pEGFP-rDNA or pEGFP- rDNA/Turbo Fect (X40).*

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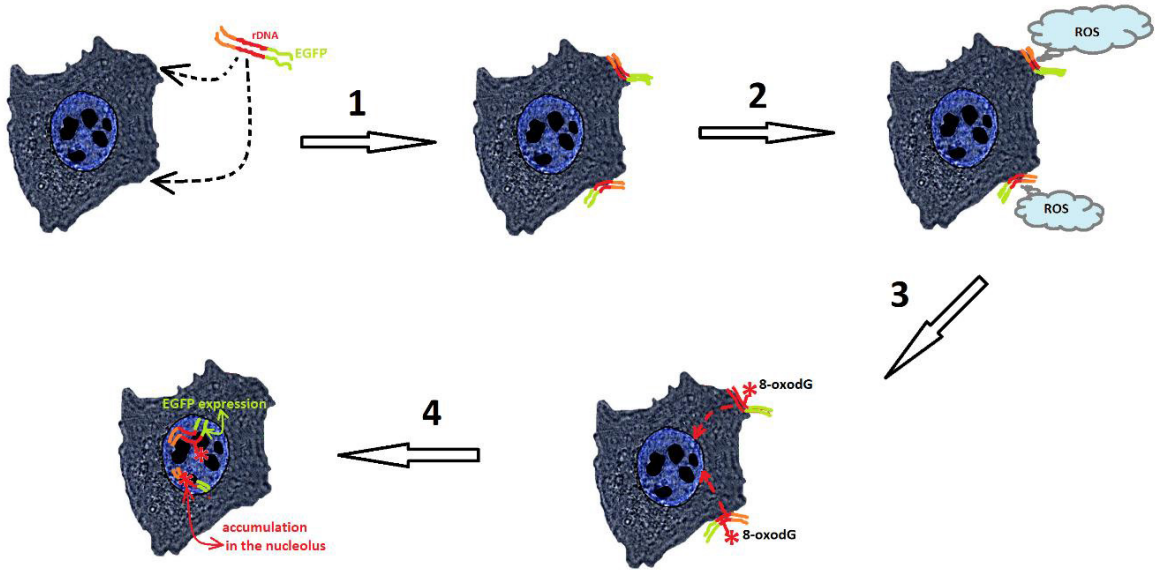
Figure 8



*A summary of events developing in MCF7 cells exposed to GC-DNA (pEGFP-rDNA as an example) which contains the genes that can be transcribed. (**1**) GC-DNA approaches and interacts with the cell surface. (**2**) GC-DNA stimulates the burst of ROS at the site of contact with the cell. ROS level is independent of the presence of easily oxidizable motives. (**3**) The easily oxidizable bases of the rDNA (or other human easily oxidizable DNA sequences) is oxidized. GC-DNA without an easily oxidizable rDNA insert is oxidized to a lesser extent. (**4**) Oxidized GC-DNA is transfected into the nucleus of the cell and can be expressed (EGFP gene in pEGFP-rDNA molecules with intact promoter is expressed). GC-rDNA molecules accumulate in the nucleolus.*

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