MicroRNA-21 Modulates the Levels of Reactive Oxygen Species Levels by Targeting SOD3 and TNFα

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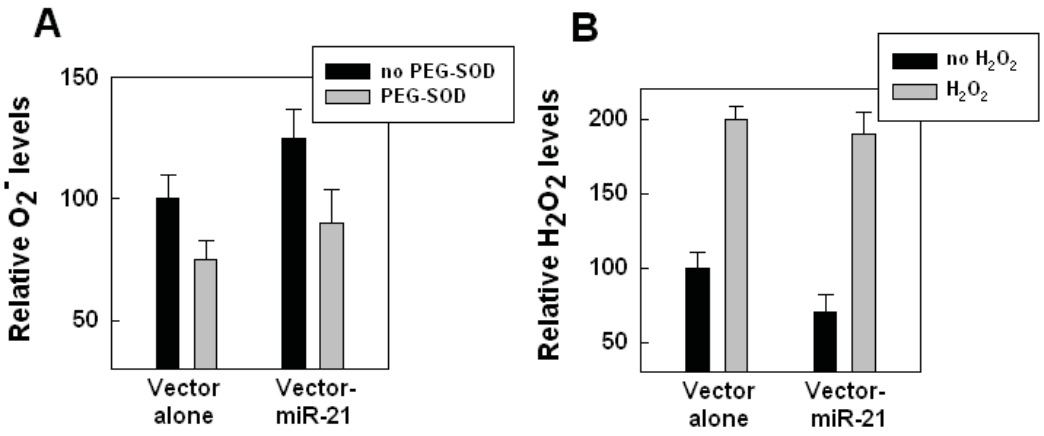
Supplementary Information includes Table S1, Figure S1-S10 and references.

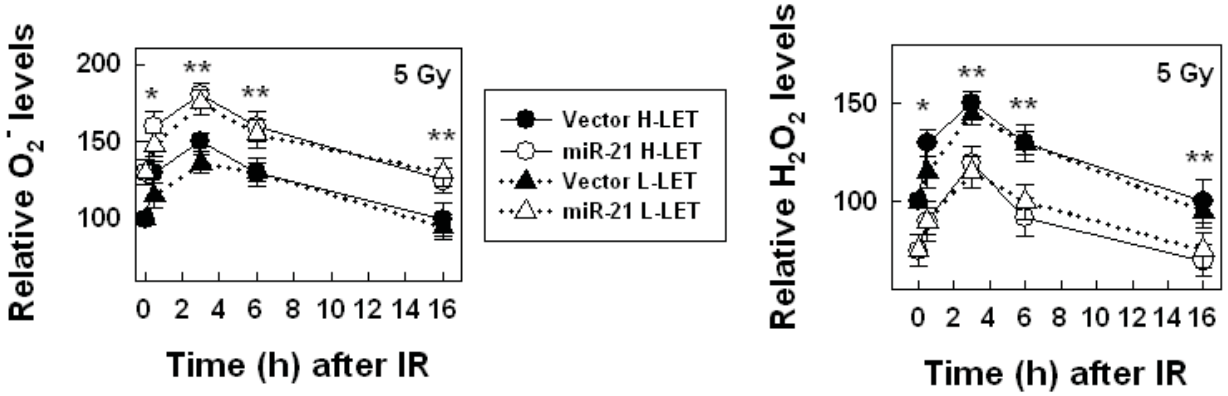
Supplementary Table S1. Sequences of the primers used in plasmid instruction or in real-time PCR

Primer name	Sequence 5'-3'	Remark
has-miR-21-f:	gcatctagAGGAGCATTATGAGCATTATGTCAGA	miRNA expression
has-miR-21-r	ataggatccCAAAAGACTCTAAGTGCCACCA	miRNA expression
SOD3-3′UTR-f	ccgctcgagAGCACTCAGAGCGCAAGAAG	Luciferase Assay
SOD3-3′UTR-r	cggactagtGGGAAGATCGTCAGGTCAAA	Luciferase Assay
TNFa-3′UTR-f	ccgctcgagTGCTGCAGGACTTGAGAAGA	Luciferase Assay
TNFa-3′UTR-r	cggactagtTCTCGCCACTGAATAGTAGGG	Luciferase Assay
SOD3-3′UTR-m21d**-f	AGACCCTCCTTCCCCACCCTGAGACTCCCGCCTTTGAC	Luciferase Assay
SOD3-3′UTR-m21d**-r	GTCAAAGGCGGGAGTCTCAGGGTGGGGAAGGAGGGTCT	Luciferase Assay
TNFa-3′UTR-m21d**-f	GCCTTCTTTTGATTATGTTTTTTAAAATATTTATCTAAA CAATGCTGATTTGGT	Luciferase Assay
TNFa-3′UTR-m21d**-f	ACCAAATCAGCATTGTTTAGATAAATATTTTAAAAAACA TAATCAAAAGAAGGC	Luciferase Assay

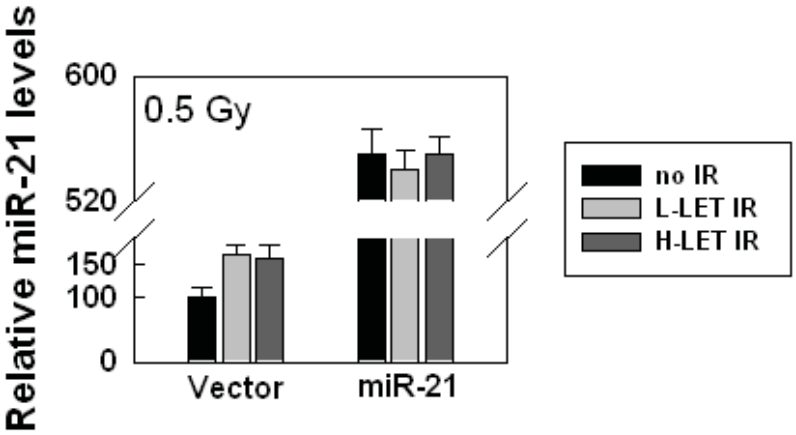
m21d**: primers for deleting miR-21 binding site on gene 3′ UTR



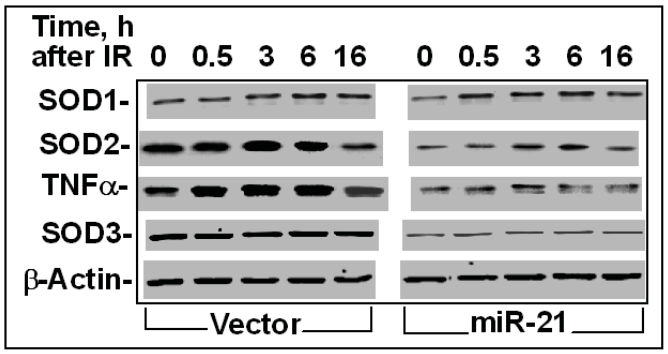
Supplementary Figure S1. Flow cytometer measuring O^-_2 or H_2O_2. A. The cells (transfected with vector alone or the vector encoding miR-21, $2x10^6$) were dyed with 1 ml of 10 μM Dihydroethidium (DHE) (Sigma) and 100 units of PEG-SOD in PBS in a 37°C incubator for 30 min. The samples were measured with flow cytometer using a BD Cytometer machine (BD FACSCanto II). The data represent the means± SD from three experiments. B. The cells were dyed with 10 μM CM-H2DCFDA (Invitrogen) and 4 μM H_2O_2 in a 37°C incubator for 30 min. The samples were measured with a flow cytometer using a BD Cytometer machine (BD FACSCanto II). The data represent the means± SD from three experiments.



Supplementary Figure S2 The ROS level (left panel: O_2^-; right panel: H_2O_2) was measured from the cells with or without miR-21 over-expression at different times after exposure to 5 Gy high- or low-LET IR using a flow cytometer as described in METHODS. The data were mean ± SD obtained from two separated experiments, *: $p< 0.05$, **: $p< 0.01$.



Supplementary Figure S3 IR increases miR-21 levels in the cells transfected with vector alone but not in the cells over-expressed with miR-21. The miR-21 levels were measured from the cells at 3 h after exposure to 0.5 Gy low- or high-LET IR and calculated as the percentage of the miR-21 level from the vector alone-transfected cells without IR. The data were mean ± SD obtained from two separate experiments.

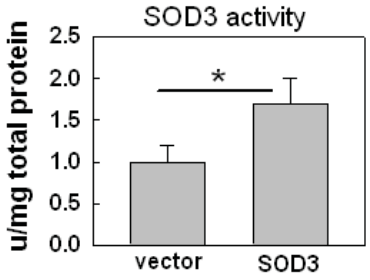


Supplementary Figure S4 The SOD levels were measured using the cells with or without miR-21 over-expression at different times after exposure to 5 Gy high- or low-LET IR with a Western blot. β-Actin was used as an internal control.

SOD3-3'UTR sequences and miR-21 target region

GCGCGGCCCCCACCCGGCGGCGGCCAGGGACCCCCGAGGCCCCCCTCTGCCTTTGAGCTTCTCCTCT
GCTCCAACAGACACCCTCCACTCTGAGGTCTCACCTTCGCCTTTGCTGAAGTCTCCCCGCAGCCCTC
TCCACCCAGAGGTCTCCCTATACCGAGACCCACCATCCTTCCATCCTGAGGACCGCCCCAACCCTCG
GAGCCCCCCACTCAGTAGGTCTGAAGGCCTCCATTTGTACCGAAACACCCCGCTCACGCTGACAGCC
TCCTAGGCTCCCTGAGGTACCTTTCCACCCAGACCCTCCTTCCCCACCCCATAAGCCCTGAGACTCC
CGCCTTTGACCTGACGATCTTCCCCCTTCCCGCCTTCAGGTTCCTCCTAGGCGCTCAGAGGCCGCTC
TGGGGGGTTGCCTCGAGTCCCCCCACCCCTCCCCACCCACCACCGCTCCCGCGGCAAGCCAGCCCGT
GCAACGGAAGCCAGGCCAACTGCCCCGCGTCTTCAGCTGTTTCGCATCCACCGCCACCCCACTGAGA
GCTGCTCCTTTGGGGGAATGTTTGGCAACCTTTGTGTTACAGATTAAAAATTCAGCAATTCAGTAAA
AAAAAAAAAAAAAAA
(618 bp in 3'UTR)

Supplementary Figure S5 The sequence of SOD3 3'UTR contains a potential miR-21 binding site (underlined). The red background indicates the seed match sequence with miR-21.

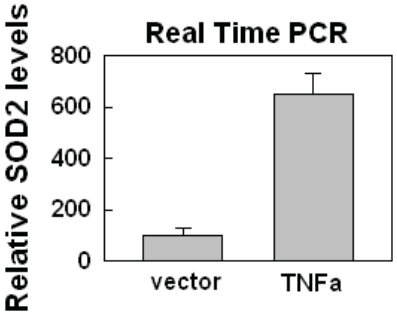


Supplementary Figure S6 SOD3 activity was measured based on a previous publication (1). Briefly the cells transfected with the vector alone or with the vector encoding SOD3 were lysed with a buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1 mM $MgCl_2$)**.** The activity of SOD3 was determined after separation with Con A–Sepharose. The results are repeated two times and the data presented as mean±SD, and the values are expressed as units per milligram of total protein. *$P < 0.05$.

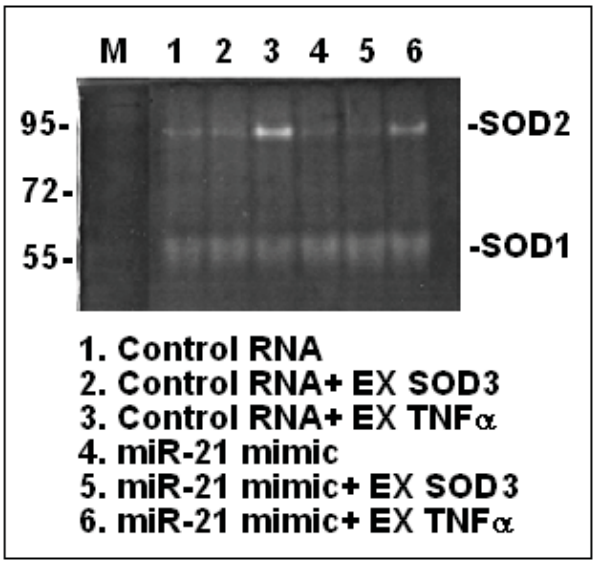
TNFa-3'UTR sequences and miR-21 target region

AGGAGGACGAACATCCAACCTTCCCAAACGCCTCCCCTGCCCCAATCCCTTTATTACCCCCTCCTTC
AGACACCCTCAACCTCTTCTGGCTCAAAAAGAGAATTGGGGGCTTAGGGTCGGAACCCAAGCTTAGA
ACTTTAAGCAACAAGACCACCACTTCGAAACCTGGGATTCAGGAATGTGTGGCCTGCACAGTGAAGT
GCTGGCAACCACTAAGAATTCAAACTGGGGCCTCCAGAACTCACTGGGGCCTACAGCTTTGATCCCT
GACATCTGGAATCTGGAGACCAGGGAGCCTTTGGTTCTGGCCAGAATGCTGCAGGACTTGAGAAGAC
CTCACCTAGAAATTGACACAAGTGGACCTTAGGCCTTCCTCTCTCCAGATGTTTCCAGACTTCCTTG
AGACACGGAGCCCAGCCCTCCCCATGGAGCCAGCTCCCTCTATTTATGTTTGCACTTGTGATTATTT
ATTATTTATTTATTATTTATTTATTTACAGATGAATGTATTTATTTGGGAGACCGGGGTATCCTGGG
GGACCCAATGTAGGAGCTGCCTTGGCTCAGACATGTTTTCCGTGAAAACGGAGCTGAACAATAGGCT
GTTCCCATGTAGCCCCCTGGCCTCTGTGCCTTCTTTTGATTATGTTTTTTAAAATATTTATCTGATT
AAGTTGTCTAAACAATGCTGATTTGGTGACCAACTGTCACTCATTGCTGAGCCTCTGCTCCCCAGGG
GAGTTGTGTCTGTAATCGCCCTACTATTCAGTGGCGAGAAATAAAGTTTGCTTAGAAAAGAA
(799 bp in 3'UTR)

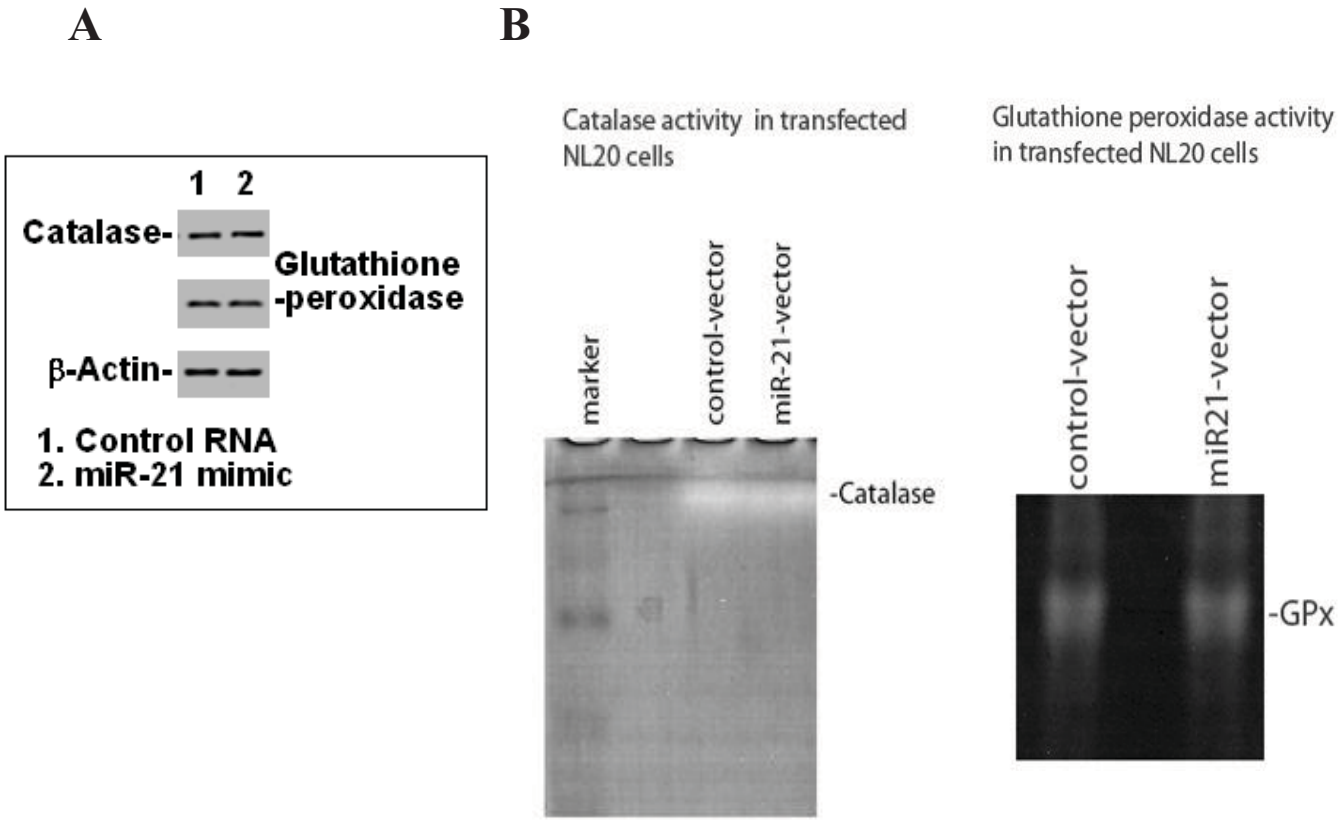
Supplementary Figure S7 The sequence of TNFα 3'UTR contains a potential miR-21 binding site (underlined). The red background indicates the seed match sequence with miR-21.



Supplementary Figure S8 The SOD2 mRNA levels were measured using the cells with stable miR-21 over-expression and up-regulated with TNFα (without 3'-UTR) with a real-time PCR assay. The data were mean ± SD from two separate experiments with 6 repeats.



Supplementary Figure S9 SOD activity. SOD activity assay was performed using 10% acrylamide native mini gel according to the previously published methods (2, 3). Briefly, 25 μg of total protein from each cell sample was loaded. The electrophoresis was run at 100V for 3 hr. The gel was washed and soaked 0.2 mg/ml of nitro blue tetrazolium (Sigma). The gel was transferred to a buffer containing 28 mM Temed (Bio-Rad,), 0.28 μM riboflavin (Sigma), and 36 mM potassium phosphate (pH 7.4) for 15 min. The gel was illuminated with a fluorescent light for 20 min. Superoxide dismutase (SOD)-active areas appeared as clear zones on a blue-violet background.



Supplementary Figure S10 miR-21 does not affect the levels and activities of Catalase and Glutathione peroxidase (GPx). (A) The levels of catalase and glutathione peroxidase measured by Western blot. Whole cell lyses were prepared from the NL20 cells transfected with the control RNA or miR-21 mimic. The cells were collected at 48 h after transfection. The antibody against catalase or β-Actin was purchased from Santa Cruz Biotechnology and the antibody against glutathione peroxide was purchased from Cell Signaling. (B) The activities of Catalase and Glutathione peroxidase was measured according to a protocol as described previously (3). Lef panel, NL20 cells transfected with vector alone or the vector encoding miR-21 were subjected to 8% native gel electrophoresis (100 μg protein per well) and stained. Achromatic (clear) bands represent areas of catalase enzyme activity. Right panel, cells were harvested and the protein lysates (250 μg) were loaded onto gels (8%) and subjected to GPx native gel activity analysis.

References:

1. Fukai T, Galis ZS, Meng XP, Parthasarathy S, Harrison DG. Vascular expression of extracellular superoxide dismutase in atherosclerosis. J Clin Invest. 1998;101:2101-11.
2. Lygren ST, Closs O, Bercouvier H, Wayne LG. Catalases, peroxidases, and superoxide dismutases in Mycobacterium leprae and other mycobacteria studied by crossed immunoelectrophoresis and polyacrylamide gel electrophoresis. Infect Immun. 1986;54:666-72.
3. Weydert CJ, Cullen JJ. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protocols. 2010;5:51-66.