ONLINE SUPPLEMENT

MATERIALS

Mouse vascular smooth muscle cells. A. Corsini, S. Bellosta and M. Canavesi have prepared primary VSMCs (mVSMC) from the PPARα$^{-/-}$ and p16$^{-/-}$ mice, kindly provided by F. Gonzalez[1] and P. Krimpenfort[2], respectively.

Reagents and plasmids. Wy14,643 and fenofibric acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO) and Toronto Research Chemicals. Inc. (CA), respectively. GW7647[3] was kindly provided by P. J. Brown from GlaxoSmithKline (Research Triangle Park, North Carolina, USA). 5'-deletion series of human TERT promoter constructs were kindly provided by S. Kyo (Kanazawa University School of Medicine, Japan).[4] The reporter constructs bearing site-directed mutations of the E2F consensus elements from -267 to the immediate upstream part of the translation start codon of the human TERT gene were a kind gift from T.K. Kim (Korea Advanced Institute of Sciences and Technology, Taejon, Korea).[5] The p16 expression vector cloned into pcDNA3 was a generous gift of C. Labrie (Laval University Medical Research Center, Quebec, Canada). The pSG5-hPPARα plasmid and recombinant Ad-GFP and Ad-GFP/PPARα adenovirus were described previously.[6, 7] The adenovirus coexpressing TERT and green fluorescent protein (Ad-GFP/TERT) was kindly provided by M.D. Schneider (Baylor College of Medicine, Houston, TX, USA).[8] The adenovirus overexpressing human E2F-1 (Ad-E2F-1) was provided by W.R. MacLellan (University of California, Los Angeles).[9, 10]

siRNA. siRNAs were either from Dharmacon (L-003434-00) for human PPARα, or from Ambion for p16 (42635), E2F-1 (114509), or TERT (114509). The non-silencing siRNA oligonucleotide from Qiagen, which does not target any known mammalian gene, was used as a negative control.

Antibodies. Antibodies for TERT (ab5181, Abcam) and the nuclear factor TFIIB (sc-274, Santa Cruz Biotechnology) were used in Western blot assays. Antibodies against underphosphorylated pRB (554164, Pharmingen), p107 (sc-318, Santa Cruz Biotechnology), p130 (MS-866, Neomarker) or E2F-1 (sc-251, Santa Cruz Biotechnology) were used in Chromatin immunoprecipitation (ChIP) assays.

Online Table I. Oligonucleotides used in this study.

Name	Use	Sequence	Ref.
hTERT	Real-time RT-PCR	F : 5'-TTCCTGCACTGGCTGATGAG-3' R: 5'-CCGGTAGAAAAAGAGCCTGTTC -3'	
mTERT	Real-time RT-PCR	F: 5'-GGCTCTTCTTCTACCGTAAG-3' R: 5'-TGATGCTTGACCTCCTCTTG-3'	11
hPPARα	Real-time RT-PCR	F: 5'-GGTGGACACGGAAAGCCCAC-3' R: 5'-GGACCACAGGATAAGTCACC-3'	12
hp16	Real-time RT-PCR	F: 5'-GAGCAGCATGGAGCCTTC-3' R: 5'-GGCCTCCGACCGTAACTATT -3'	
hE2F-1-1 (Fig. 4D)	Real-time RT-PCR	F: 5'-CAAGAAGTCCAAGAACCACATCC-3' R: 5'-AGATATTCATCAGGTGGTCCAGC-3'	13
hE2F-1-2 (Fig. S2)	Real-time RT-PCR	F: 5'-GAACTGAGGCCTGGGTGATTT-3' R : 5'-CCACCCATGGCTGTCAGTC-3	14
hTBP	Real-time RT-PCR	F: 5'-GGAGAGTTCTGGGATTGTACCGC-3' R: 5'-ATATTCGGCGTTTCGGGCAC-3'	
mTFIIB	Real-time RT-PCR	F: 5'- CTCTCCCAAGAGTCACATGTCC-3' R: 5'-CAATAACTC GGTCCCCTACAAC-3'	
mACO	Real-time RT-PCR	F: 5'-ACGTCTTGGATGGTAGTCCG-3' R: 5'-TAACGCTGGCTTCGAGTGAG-3'	15
hTERTE2F1	Real-time ChIP PCR	F: 5'-GGCCGATTCGACCTCTCT-3' R: 5'-AAGGTGAAGGGGCAGGAC-3'	
hTERTcontrol	Real-time ChIP PCR	F: 5'-TACAGCATCCCTGCAAGGCCTC-3' R: 5'-GACTTCCAGCCCAGCAGTAG-3'	
mTERTE2F1	Real-time ChIP PCR	F: 5'-CTTTGCTTGCCCAAACCTCGC-3' R: 5'-GGTTGCTGGATGTGTTGGAG-3'	
mTERTcontrol	Real-time ChIP PCR	F: 5'-GTGAGTTGAGATGATGCTCTGG-3' R: 5'-CGACCATACACTCAGATCCC-3'	

ONLINE FIGURES

Online Figure I. E2F-1 overexpression increases TERT mRNA expression and telomerase activity in hVSMCs. G_0/G_1 arrested hVSMCs were infected for 3h with 25 PFU/cell of an adenovirus expressing either E2F-1 (Ad-E2F-1) or GFP (Ad-GFP). Cells were harvested 36h after infection for Western blot analysis of nuclear E2F-1 or TFIIB (used to control for equal protein loading) expression (A), measurement of TERT mRNA (B), and telomerase activity (C) levels. Figure A depicts a representative Western Blot from three independently performed experiments. Values in Figures B and C are expressed as mean ± SEM (n=3; # $P < 0.05$ *vs.* Ad-GFP infected cells).

Online Figure II. Activation of PPARα/p16 pathway modulates transcriptional complexes formed with E2F and pRB family proteins on the proximal TERT gene promoter in mouse VSMCs (mVSMCs). Primary aortic VSMCs (mVSMCs) were prepared from 8- to 12-week-old littermate wild-type, PPARα$^{-/-}$ or p16$^{-/-}$ mice on a C57Bl/6J mice as described.[7] mVSMCs were subjected to G_0/G_1 cell synchronization by starvation in medium supplemented with 0.4% FBS for three days. mVSMCs were then reincubated in routine growth medium containing 20% FBS supplemented with Wy14,643 (10 μM) or vehicle (Me2SO). After 72h, cells were harvested and soluble chromatin was prepared. Chromatin immunoprecipitations were performed with the indicated antibodies followed by real-time PCR amplification using primer pairs that cover the E2F element in the proximal mouse TERT gene promoter (mTERTE2F1, Table I). Results are representative of three independent experiments and are expressed as mean ± SEM (# $P < 0.05$ *vs.*

vehicle treated mVSMCs). Representative ethidium bromide-stained agarose gels showing PCR products obtained after 35 elongation cycles are shown. Negative controls include PCRs performed with mTERTE2F1 oligonucleotides following immunoprecipitation with nonimmune IgG, or with primers covering a distal region of TERT gene localized at -368/-230 of E2F element (mTERTcontrol, Table I, data not shown).

Online Figure III. Overexpression of TERT prevents the inhibitory effect of PPARα activation by fenofibric acid (FA) on $G_1 \rightarrow S$ cell cycle progression. G_0/G_1 arrested hVSMCs were infected for 6h with an adenovirus expressing GFP (Ad-GFP) or TERT (Ad-GFP/TERT) at 100 PFU/cell. Following infection, cells were pretreated for 24h with vehicle (Me_2SO) or FA (300 μM) and reincubated in growth medium. After 24h, cells were harvested for FACS analysis to characterize cell cycle distribution. The mean from three experiments performed with different cell preparations is shown.

Online Figure IV. Hepatic acyl-CoA oxidase (ACO) mRNA levels in Sv/129 mice 9 days after treatment with fenofibrate or gemfibrozil. Sv/129 mice were fed a diet supplemented with fenofibrate (0.05%, n=8; 0.02%, n=6) or gemfibrozil (0.5%, n=7) for one week prior to endovascular femoral artery wire injury. Mice fed standard chow diet served as control group (n=8). Livers were isolated for mRNA extraction simultaneously to femoral artery tissues two days after vascular injury. ACO mRNA expression was analyzed by real-time RT-PCR and normalized to TFIIB mRNA expression. Data are

presented as mean ± SD percent increase relative to untreated control mice (# $P < 0.05$ *vs.* untreated mice, * $P < 0.05$ between groups as indicated).

Online Figure V. siRNA-mediated down-regulation of PPARα and p16 expression increases TERT gene expression and telomerase activity in hVSMCs. Quiescent hVSMCs plated in 6-well plates were transfected either with PPARα (2 μg, A) or p16 (2.8 μg, B) siRNAs or the equivalent amount of nonsilencing siRNA. After 4h, routine growth medium was added to the plates. Cells were harvested after 24h for quantitative RT-PCR and telomerase activity assays as indicated. Data represents the mean ± SEM (n=3; # $P < 0.05$ *vs.* non-silencing siRNA-transfected hVSMCs).

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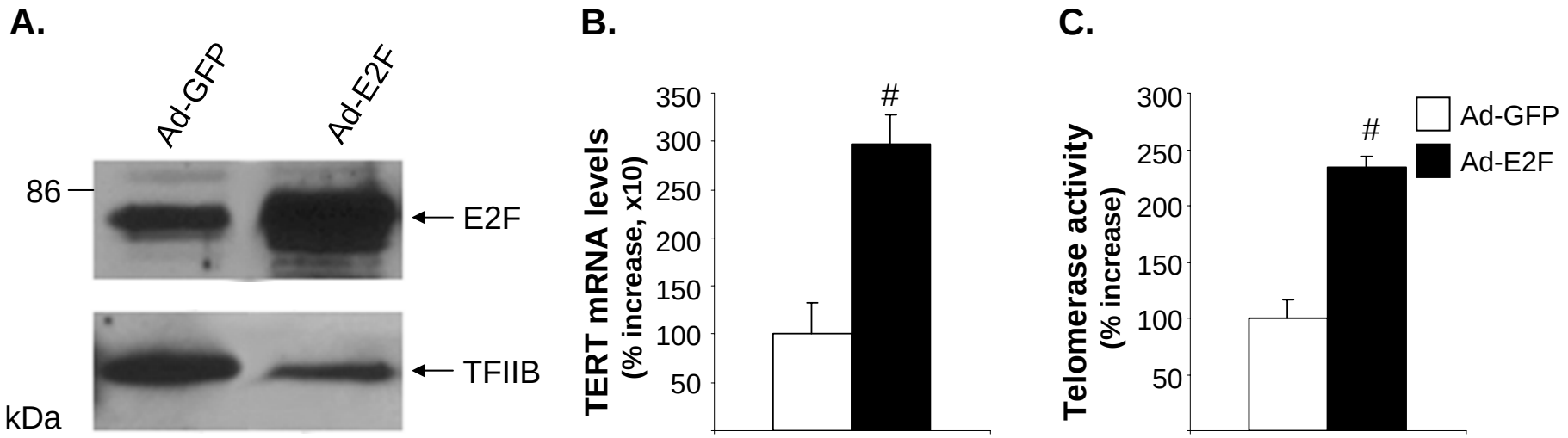
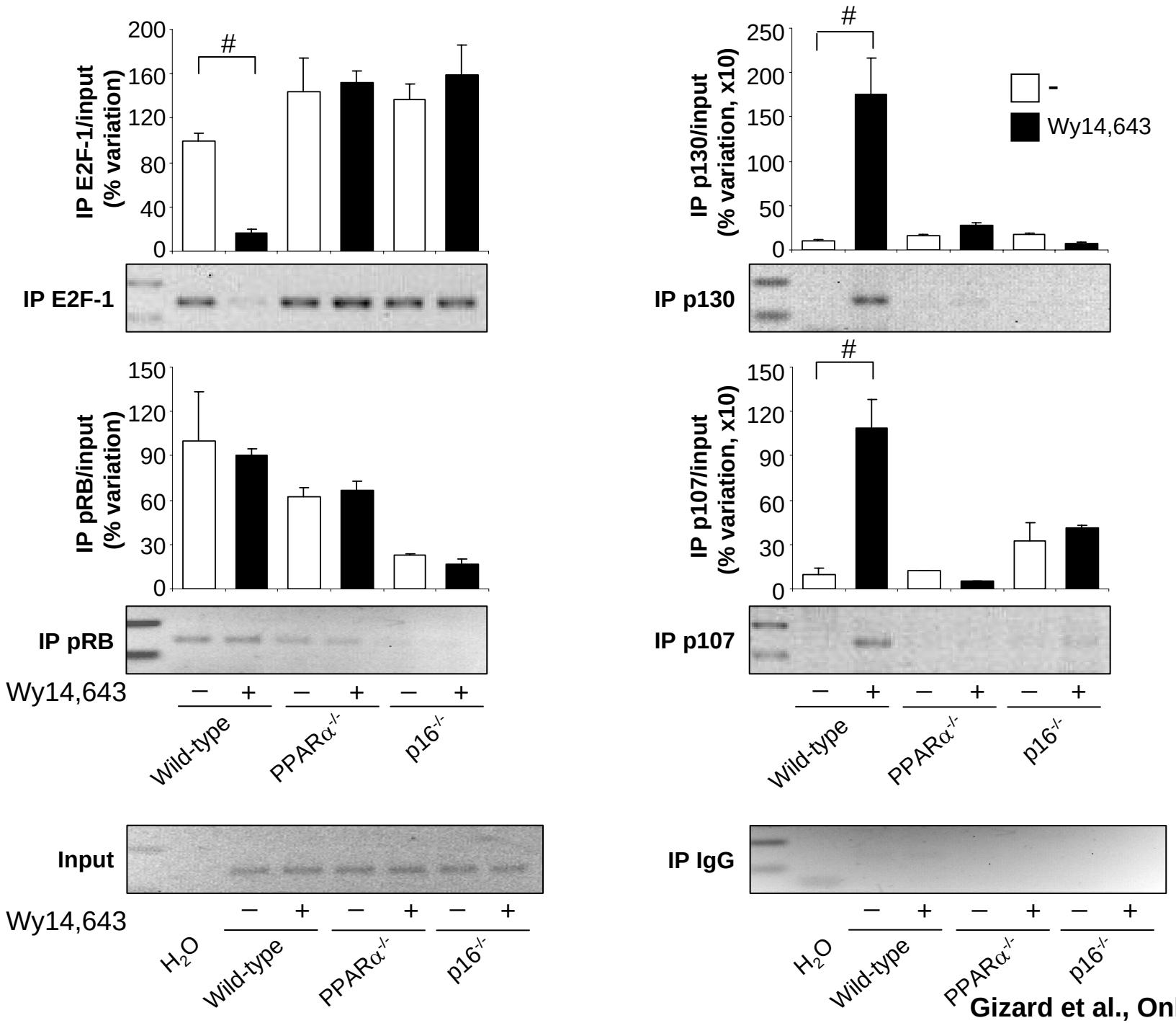


Gizard et al., Online Figure I



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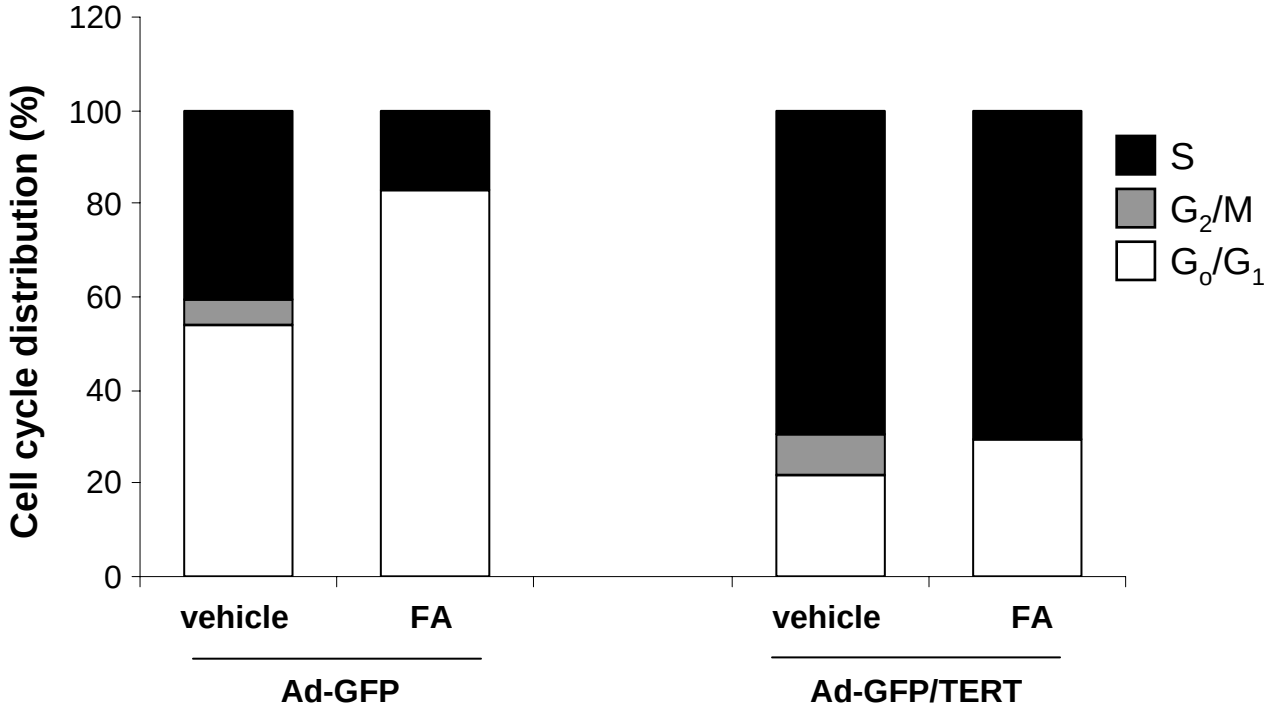

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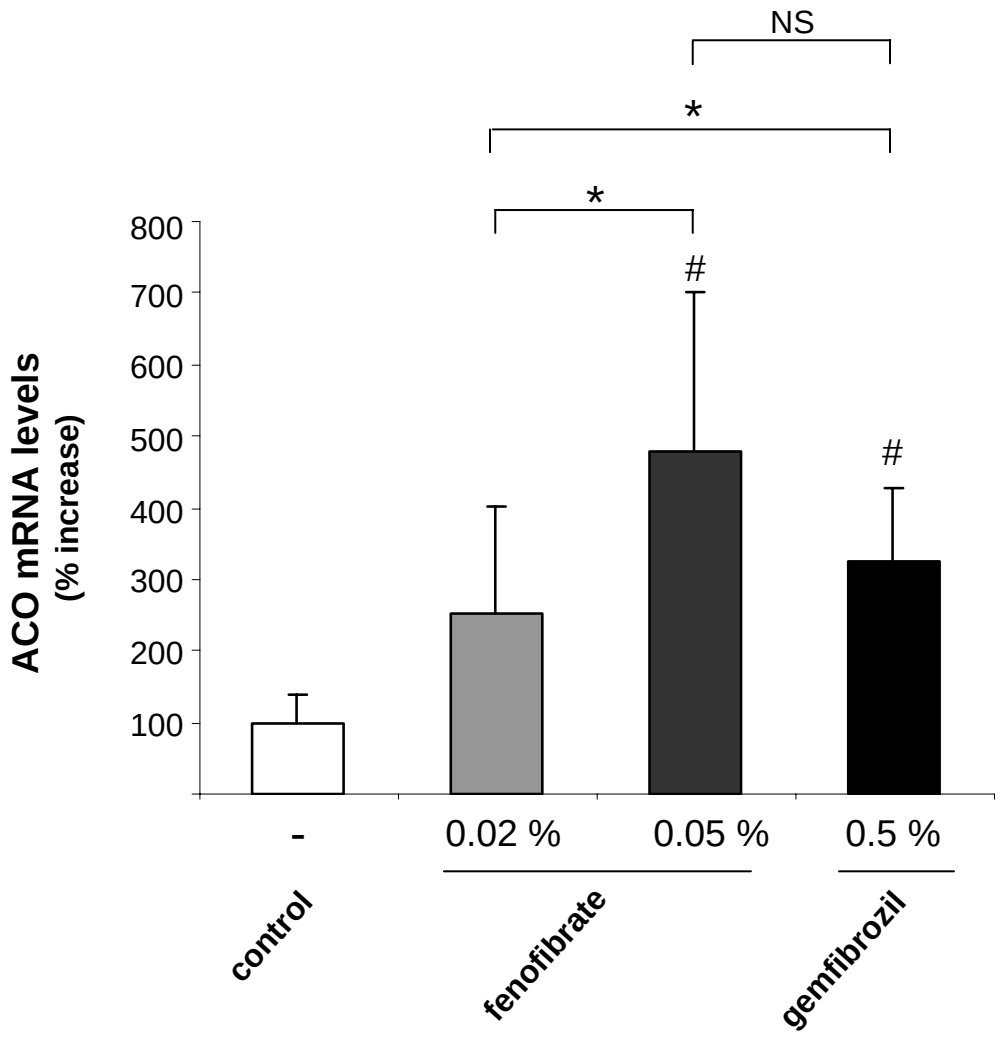

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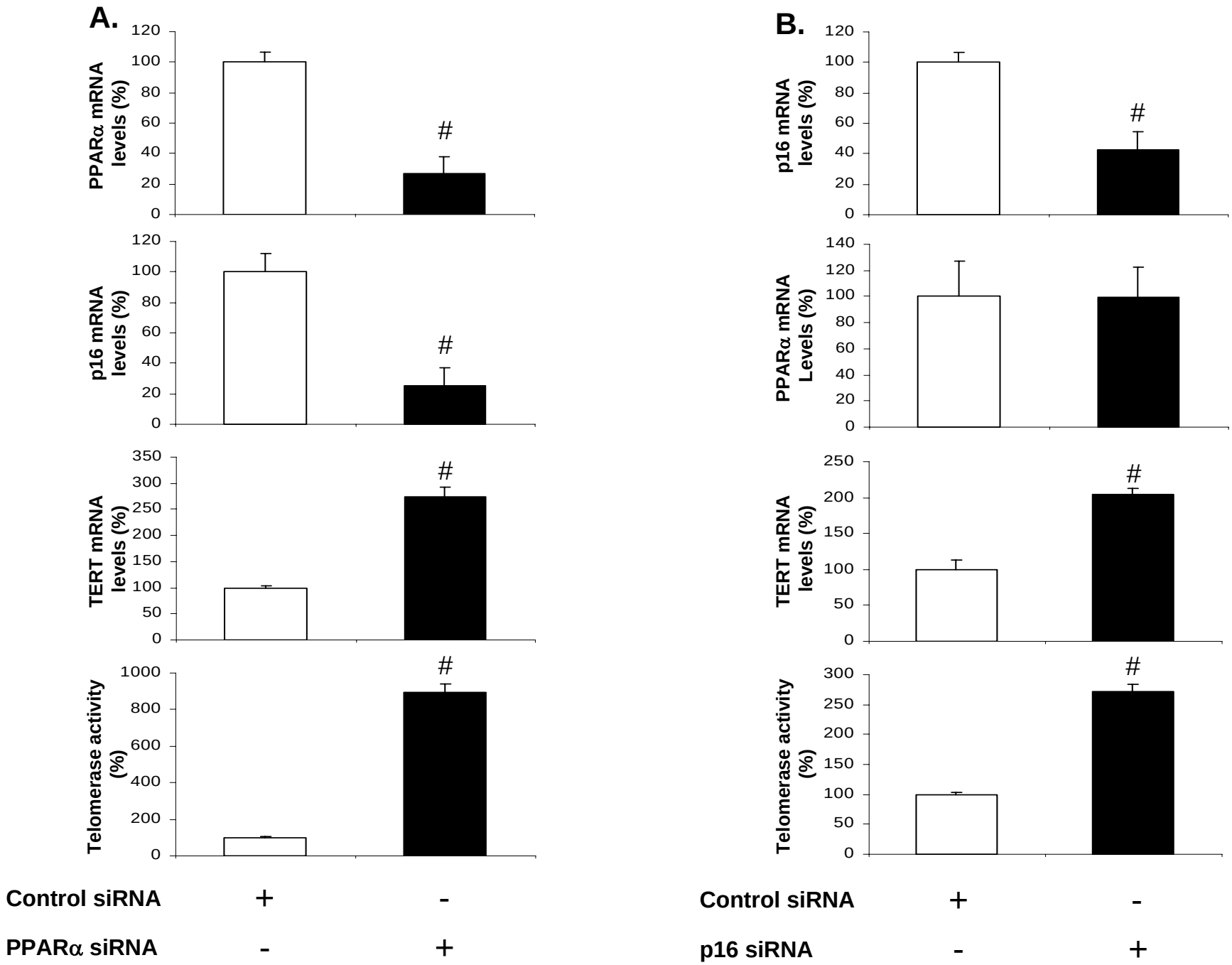

Gizard et al., Online Figure V