SUPPLEMENTARY MATERIAL

p19^Arf deficiency reduces macrophage and vascular smooth muscle cell apoptosis and aggravates atherosclerosis

Herminia González-Navarro 1, Yafa Naim Abu Nabah 1, Ángela Vinué 1, María J. Andrés-Manzano 2, Manuel Collado 3, Manuel Serrano 3, Vicente Andrés 1,2,4

1 Vascular Biology Unit, Department of Molecular and Cellular Pathology and Therapy, Instituto de Biomedicina de Valencia (IBV), Spanish Council for Scientific Research (CSIC), 46010 Valencia, Spain

2 Laboratory of Molecular and Genetic Cardiovascular Pathophysiology, Department of Atherothrombosis and Cardiovascular Imaging, Spanish National Cardiovascular Research Center (CNIC), 28029 Madrid, Spain

3 Spanish National Cancer Research Center (CNIO), 28029 Madrid, Spain

4 Address for correspondence:

Vicente Andrés, Spanish National Cardiovascular Research Center (CNIC), Melchor Fernández Almagro 3, 28029 Madrid (Spain), Phone: +34-914531200, Fax: +34-914531265, e-mail: vandres@cnic.es.
ADDITIONAL METHODS

**Mouse genotyping.** Mice were genotyped by PCR using Taq polymerase (1 unit, Biotools). For p19Arf genotyping the PCR reaction consisted of a first step at 94°C (3 min), followed by 35 cycles of amplification (94°C, 1 min; 60°C, 1 min; 72°C, 1 min), and a final extension of 10 min at 72°C. The wild-type allele was detected as a band of 415 bp amplified using the primers ARF1 (5’-AGTACAGCAGCCGGAGCATGG-3’) and ARF2 (5’-TTGAGGAGGACCGTGGAAGCCG-3’). The KO allele was detected as a band of 250 bp amplified using the primers Neo-2 (5’-ACCACACTGCTCGACATTTGGG-3’) and ARF2. The PCR reaction for apoE genotyping consisted of a first step at 94°C (2 min), followed by 38 cycles of amplification (94°C, 20”; 62°C, 30”; 72°C, 30”), and a final extension of 10 min at 72°C. The wild-type allele was detected as a band of 155 bp amplified using the primers 180 (5’-GCCTAGCGAGGAGAGCCG-3’) and 181 (5’-TGTGACTTGGAGCTCTGCAGC-3’). The KO allele was detected as a band of 245 bp amplified using the primers 180 and 182 (5’-GCGCCCCCGACTGATCT-3’).

**Gene expression analysis by quantitative real-time PCR (qPCR).** RNA from aortic tissue of mice fed atherogenic diet for 4 and 9 weeks and from mouse VSMCs was obtained in a TissueLyser (Qiagen) using TRIzol Reagent (Invitrogen). RNA purity and concentration was determined by the A260/280 ratio. RNA (0.5-1μg) was retro-transcribed and amplified with SuperScript III First Strand Synthesis Supermix and Platinum SYBR Green qPCR Supermix-UDG with Rox dye (both from Invitrogen). Reactions were run on a thermal Cycler 7500 Fast System and results were analyzed with the software provided by the manufacturer (Applied Biosystems). The following primers (Forward: Fw; Reverse: Rv) were used: cyclophilin (Fw: 5’-
TGGAGAGCACCAAGACAGACA-3’ and Rv 5’-TGCCGGAGTCGACAATGAT-3’); $p15^{INK4b}$ (Fw 5’-AGATCCCAACGCCCTGAAC-3’ and Rv 5’-CCCATCATCATGACCTGGATT-3’); $p16^{INK4a}$ (Fw: 5’-CGTACCCCGATTCAAGTGTAT-3’ and Rv 5’-TTGAGCAGAAGGCTGCTACGT-3’); c-myc (Fw 5’-GCCCCCAAGGTAGTGATCGG-3’ and Rv 5’-GTG CTC GTC TGC TTG AAT GG-3’).
Figure S1. p19^{Arf} inactivation in apoE^{-/-} mice does not affect proliferation at early stages of atherosclerosis. Aortic root cross-sections from mice fed atherogenic diet for 4 weeks were stained with anti-Ki67 antibody to quantify cell proliferation in the atherosclerotic lesions (as percentage of positive cells). Under this dietary regimen, atheromas were smaller than in the mice challenged with atherogenic diet for 9 weeks, but the percentage of neointimal Ki67-immunoreactive cells was higher (confer Fig.4C). Images were captured at a magnification of 1000X. Statistical analysis was carried out using the Student’s t-test.
Figure S2. p16\(^{\text{INK4a}}\) and p15\(^{\text{INK4b}}\) mRNA expression in aortic arch and cultured VSMCs. qPCR was carried out using the primers described in this online supplement. (A) The mRNA level of p16\(^{\text{INK4a}}\), but not that of p15\(^{\text{INK4b}}\), was markedly increased in the aortic arch of apoE\(^{-/-}\)p19\(^{-/-}\) mice compared to apoE\(^{-/-}\) mice fed atherogenic diet for 4 and 9 weeks. (B) Analysis in aortic VSMCs revealed that the levels of p16\(^{\text{INK4a}}\) and p15\(^{\text{INK4b}}\) mRNA did not reach statistically significant differences between apoE\(^{-/-}\) and apoE\(^{-/-}\)p19\(^{-/-}\) cells. Expression in apoE\(^{-/-}\)p19\(^{-/-}\) aortic arch and VSMCs is expressed relative to apoE\(^{-/-}\) mRNA levels (=1). p16\(^{\text{INK4a}}\) and p15\(^{\text{INK4b}}\) mRNA expression was normalized with the respective expression of the endogenous cyclophilin gene. Statistical analysis was carried out using 2-way ANOVA (A) and Student’s t-test (B). NS: not significant.
Figure S3. c-myc mRNA expression by qPCR analysis in aortic VSMCs and aortic arch of mice. (A) Expression analysis by qPCR shows increased mRNA levels of c-myc in aortic VSMCs lacking p19Arf. (B) mRNA levels of myc are increased in the aortic arch of apoE−/−p19−/− mice compared to apoE−/− mice when fed atherogenic diet for 4 weeks but not 9 weeks. Expression in VSMCs and aortic arch from apoE−/−p19−/− is expressed relative to that in apoE−/− controls (set as 1) after normalization with the respective level of cyclophilin expression. Statistical analysis was carried out using Student’s t-test (A) and 2-way ANOVA (B).