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ORIGINAL ARTICLE

Expression and potential role of apolipoprotein D on the death–survival balance of human colorectal cancer cells under oxidative stress conditions

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Abstract
Purpose Inverse correlations of apolipoprotein D (ApoD) expression with tumor growth have been shown, therefore proposing ApoD as a good prognostic marker for diverse cancer types, including colorectal cancer (CRC). Besides, ApoD expression is boosted upon oxidative stress (OS) in many pathological situations. This study aims at understanding the role of ApoD in the progression of human CRC.
Methods Samples of CRC and distant normal tissue (n=51) were assayed for levels of lipid peroxidation, expression profile of OS-dependent genes, and protein expression. Three single-nucleotide polymorphisms in the ApoD gene were analyzed (n=139), with no significant associations found. Finally, we assayed the effect of ApoD in proliferation and apoptosis in the CRC HT-29 cell line.
Results In CRC, lipid peroxides increase while ApoD messenger RNA and protein decrease through tumor progression, with a prominent decrease in stage I. In normal mucosa, ApoD protein is present in lamina propia and enteroendocrine cells. In CRC, ApoD expression is heterogeneous, with low expression in stromal cells commonly associated with high expression in the dysplastic epithelium. ApoD promoter is basally methylated in HT-29 cells but retains the ability to respond to OS. Exogenous addition of ApoD to HT-29 cells does not modify proliferation or apoptosis levels in control conditions, but it promotes apoptosis upon paraquat-induced OS.
Conclusion Our results show ApoD as a gene responding to OS in the tumor microenvironment. Besides using ApoD as marker of initial stages of tumor progression, it can become a therapeutic tool promoting death of proliferating tumor cells suffering OS.

Keywords Lipocalin · Lipid peroxidation · Apoptosis · Predictive marker · DNA methylation · Colorectal cancer stages

MDG and DS contributed equally to this work.

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Introduction

Colorectal cancer (CRC) is the second most common cancer in developed countries, being the third in frequency worldwide. CRC counts as the second most frequent cancer in women, following breast cancer, while it is the third most common cancer in men, after those from prostate and lung.

In terms of causes underlying CRC, well-known risk factors are hereditary genetic predisposition, age, inflammatory colon diseases, and diet composition [1]. A correlation

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between inflammation and CRC development has been observed in both chronic inflammation processes and inflammatory bowel disease. The link between them may be the increase in COX-2 and nuclear factor kappa B during inflammation, which triggers the release of tumor necrosis factor alpha and interleukin-6. These signals have been associated with tumor growth [2].

Growing evidence also points to the participation of reactive oxygen species (ROS) and their reactive derivatives in the pathogenic origin and development of CRC. Inflammation and environmental factors lead to the generation of large amounts of ROS in colon tissue, inducing in turn oxidative stress (OS). ROS damages DNA, proteins, and lipids, and these factors are correlated with tumor growth [3–5]. CRC cells show an increase in lipid peroxidation byproducts that could be triggered by the increased arachidonic acid levels attained by the increased activity of COX-2 [6, 7]. The accumulation of lipid peroxidation results in cell damage and death. However, cancer cells tend to reduce the levels of the antiproliferative cytokine TGF-β1 and the lipid peroxidation adduct 4-hydroxynonenal (4-HNE) as a way to prevent apoptosis [8]. The levels of lipid peroxidation thus reflect the health state of the tissue.

Apolipoprotein D (ApoD) is a member of the Lipocalin protein family whose expression has been related to cell cycle and cancer progression because this gene is induced by growth arrest and its presence reduces cell proliferation in some cellular models [9, 10]. ApoD expression is regulated by p73 and p63, members of the p53 tumor suppressor family, both implicated in development control [11]. Many reports show an inverse correlation of ApoD expression levels with tumor growth [12], therefore proposing ApoD as a good diagnostic and prognostic marker.

Our laboratory studies the role of ApoD and its homologues in the nervous system using genetic model organisms such as the mouse and the fruit fly *Drosophila melanogaster*. Inducing OS by treatment with the ROS generator paraquat (PQ), we have demonstrated a protective function of ApoD in the organism survival. This effect was associated with a role in regulating tissue homeostasis and in maintaining low levels of lipid peroxidation [13–15]. We have recently shown that ApoD contributes to reduce inflammation levels in ROS-injured tissues and promotes the clearance of lipid peroxides in cells [16, 17].

The fact that ApoD expression is inversely correlated with the malignity of various tumors has been described, but what triggers this repression and how it is causally linked to the final outcome of the patient is still poorly understood. As mentioned above, in many instances, tumoral tissues develop OS. This fact represents a paradox, since OS is a positive stimulus triggering ApoD upregulation in other systems. An analysis of gene expression and an evaluation of oxidative stress in the tissue along the progression of CRC are here combined with studies in a cell-culture system to solve this question. Our aim is to get a deeper understanding of how ApoD has an impact in cancer progression and patient survival.

In this report, we study the expression of ApoD in different stages of cancer progression and its relationship with the levels of lipid peroxidation in CRC tissues. We also evaluate OS-dependent genes that show coregulated expression patterns with ApoD to uncover potential signaling pathways that can become targets for therapeutic intervention. Our results show a repression of ApoD gene expression in CRC, particularly in the initial stages of the disease, which correlates with an elevation of lipid peroxide adducts in the tissue. We also found several genes involved in ROS metabolism and antioxidant function that show expression patterns similar to ApoD through CRC progression stages. Finally, the role of ApoD in proliferation and apoptosis of tumor cells was studied, and the dependence of gene expression on the DNA methylation state will be discussed in the context of tumor development and progression.

Materials and methods

Patients and tumor features

Our study was conducted on a group of 51 patients, of 70.14± 11.21 years of age, surgically treated for colorectal cancer at the Surgery Department of the Hospital Clínico Universitario de Valladolid (Spain) (Table 1). The time since CRC diagnosis was 3–8 weeks. Patients receiving oncological treatment before surgery were excluded from the study. The study was approved by the Hospital Ethics Committee, in accordance with The Code of Ethics of the World Medical Association for experiments involving humans, and all subjects provided informed consent for the procedures.

The tumors belong to the histological type adenocarcinoma and were diagnosed by routine histopathological examination in the Pathology Department of the hospital.

The clinical evaluation of CRC progression involves tumor features such as size, penetration of colon tissue, lymph node, and vascular invasion, and the presence of distant metastases. We followed the classification stages (E0-IV), advocated by the American Joint Committee on Cancer, to categorize our samples.

Samples of tumor and healthy mucosa were selected, being the latter excised out of tissue at least 6 cm away of tumor edges. The samples used for biochemical and molecular biology experiments (~1 cm^3) were processed within 30–45 min after resection and either frozen in RNAlater (Ambion, USA) or snap-frozen and stored in liquid nitrogen. Samples used for immunohistochemistry were fixed in formalin and embedded in paraffin.

Table 1 Clinical data of the CRC cases studied in this work

Characteristics of patients studied		
Number of patients (*n*)		51
Gender (*n*)		
	Male	29 (56.86 %)
	Female	22 (43.14 %)
	Gender by stage (male/female)	SI (9/7), SII (8/6), SIII (4/3), SIV (8/6)
Age (years)		
	Mean age	70.14±11.21
	Age range	44–91
	<50 (*n*)	3 (5.88 %)
	>50 (*n*)	48 (94.12 %)
	≥80 (*n*)	13 (25.49 %)
	Age by stage	SI (72.25±10.36), SII (66.29±13.36), SIII (68.86±9.63), SIV (72.21±10.66)
Tumor size (mm)		
	Mean size	46.90±25.56
	Size by stage	SI (33±12.33), SII (54.21±31.96), SIII (67.86±32.64), SIV (45±16.98)
Depth of invation (n)		T1 (3), T2 (14), T3 (24), T4 (7)
	Depth of invasion by stage (*n*)	SI: T1 (3), T2 (13), T3 (0), T4 (0) SII: T1 (0), T2 (0), T3 (12), T4 (2) SIII: T1 (0), T2 (1), T3 (5), T4 (1) SIV: T1 (0), T2 (3), T3 (7), T4 (4)
TNM stage (n) (UICC)		SI (16), SII (14), SIII (7), SIV (14)
Tumor histology (n)		Nonmucinous (44), mucinous (7)
	Mucinous by stage (*n*)	SI (2), SII (1), SIII (2), SIV (2)
Grade of differentiation (n)		G1 (36), G2 (10), G3 (5), G4 (0)
	Differentiation by stage	SI: G1 (12), G2 (3), G3 (1) SII: G1 (9), G2 (3), G3 (2) SIII: G1 (4), G2 (1), G3 (2) SIV: G1 (11), G2 (3), G3 (0)
Lymph node status (n)		Negative (33), positive (18)
	Positive lymph node by stage(*n*)	SI (0), SII (0), SIII (6), SIV (12)
Prognosis after 1 year (*n*)		Died (6) (11.1 %)
	Died by stage (*n*)	SI (1), SII (1), SIII (3), SIV (1)
Control tissues		Normal colon adjacent to cancer $n=54$ for TBARS analyses, $n=16$ for qPCR anayses and $n=30$ for immunoblot analyses

Immunohistochemistry

The paraffin-embedded samples were sectioned (3 μm thick) in a rotary microtome (Thermo), and the sections were mounted onto poly-L-lysine-coated glass slides. The hydration, blocking, washes, and incubation with antibodies were performed in the automated system BOND-MAX (Leica, Spain). ApoD immunohistochemistry was performed with rabbit anti-ApoD (Novocastra, USA) diluted 1:40 in Dako Real™ antibody diluent (Dako, USA), and horseradish peroxidase (HRP)-conjugated secondary antibody (ready to use dilution in bond polymer detection solution, Novocastra, USA). Signal detection was performed with HRP-driven diaminobenzidine oxidation reaction. After dehydration, clearance, and mounting, the sections were observed with an Eclipse 90i (Nikon, Spain) microscope equipped with a DS-Ri1 (Nikon) digital camera. Images were acquired and processed with NIS-Elements BR 3.0 software (Nikon).

Evaluation of histological samples was carried out in ×200 fields of randomly selected slides and assessed by an expert pathologist. ApoD staining was categorized in four classes according to the percentage of ApoD-positive cells: 1 (0 %), 2 (<15 %), 3 (15–50 %), and 4 (>50 %).

Immunoblot analysis

Tumor and normal mucosal tissues were homogenized in lysis buffer [1 % Nonidet P-40, 0.1 % sodium dodecyl sulfate (SDS), 10 % glycerol, 1 % sodium deoxycholate, 1 mM dithiothreitol, 1 mM EDTA, 100 mM HEPES, 100 mM KCl, 10 % complete protease inhibitors (Roche, Germany) in phosphate-buffered saline (PBS)], centrifuged after 30 min at 4 °C, and the supernatants were stored at −20 °C until further use.

Protein concentration in the samples was determined with the Micro-BCA™ protein assay (Pierce, USA). Immunoblot analyses were performed with 30 μg of total protein separated by SDS polyacrylamide gel electrophoresis on a 12 % polyacrylamide gel and transferred to polyvinylidene fluoride membranes following standard procedures. As primary antibody, we used a rabbit serum antihuman ApoD (generated by Dr. C. López-Otín, Univ. Oviedo, Spain) diluted 1:1,000. As secondary antibody, we used HRP-conjugated antirabbit antibody diluted 1:2,000 (Santa Cruz Biotechnology, USA). Each blot was also incubated with HRP-conjugated anti-β-Actin antibody diluted 1:250,000 (Sigma, Spain) for normalization purposes. Membranes were developed with ECL (Millipore, USA). The integrated optical density of the immunoreactive protein bands was measured in images taken within the linear range of the charged coupled device camera (VersaDoc, BioRad, Spain) avoiding signal saturation. Mean+SD of arbitrary density units was calculated from at least duplicate blots.

Quantitative real-time RT-PCR

Tissue samples stored in RNAlater were homogenized in TRIzol (Invitrogen, Spain) with an OMNI blender (three 10-s strokes at maximum speed). RNA from tissues or cells was extracted following the TRIzol protocol. Total RNA (1 μg) was reverse-transcribed with Prime-ScriptTM (Takara, Japan) and treated with DNaseI. The complementary DNA (cDNA) obtained was used as template for quantitative reverse transcriptase PCR (qRT-PCR) amplifications. To quantify human ApoD expression, we used SYBR Green I (Takara) and the primers human ApoD-forward, 5′-CCACCCCAGTTAACCTCACA, and human ApoD-reverse, 5′-CCACTGTTTCTGGAGGGAGA. The human gene RPL18, amplified with primers RPL18-forward, 5′-CCATCATGGGAGTGGACAT, and RPL18-reverse, 5′-CACGGCC GTCTTGTTTTC, was used as a reference because neither genotype nor treatment gives a significant fold change. Amplifications were performed in five (ApoD) or four (RPL18) replicates in a Rotor-Gene RG-3000 (Corbett Research, UK) thermal cycler. Cycling conditions were as follows: 95 °C, 5 min; 40 cycles (95 °C, 30 s; 55 °C, 15 s; and 72 °C, 15 s).

To study the expression of genes related to the antioxidant response and ROS metabolism, we used qRT-PCR and a SybrGreen-based array (PAHS-065; SABiosciences, USA). Gapdh was selected as the reference gene. Amplifications were performed in quadruplicate in an ABI Prism 7900HT (Applied Biosystems, USA). Standard cycling conditions were as follows: 95 °C, 5 min; 40×(95 °C, 30 s; 60 °C, 1 min).

Changes in transcriptional expression were estimated using the $\Delta\Delta C_T$ method [18]. The following criteria were applied to our analysis. (1) Replicates with variation coefficient>2.5 % were excluded. (2) Undetermined C_T values (gene expression below detection levels) were assigned C_T= 35. Pairwise comparisons where the gene average C_T> 35 cycles in both conditions were excluded from the analysis. (3) Only transcriptional changes ≥2-fold were included in the analysis. Significant differences of gene transcriptional changes were evaluated with a Mann–Whitney *U* test, using ΔC_T of each replica. Values are expressed as mean± SEM. Only statistically significant ($P<0.05$) differences of expression are presented and discussed in the text.

ApoD polymorphism analysis

Blood samples of CRC patients included in the study (n= 139) were collected before surgery, and those of control healthy subjects (n=102) were obtained upon their approval to enter the study.

Genomic DNA was extracted from peripheral blood cells using a DNA purification system (Magna Pure, Roche). Three APOD single-nucleotide polymorphisms (SNPs) located in

chromosome 3 were genotyped. These SNPs were the following: (1) (rs5952) 15T→C, NM_001647.2:c.44T>C, NP_001638.1:p.Phe15Ser; (2) (rs1568565) –352G→A, NM_001647.2:c.124–352T>A; (3) (rs1467282) +718C→T, NM_001647.2:c.334+718T>C. Genotyping was carried out in a LightCycler 480 II (Roche) by analyzing melting curves of amplicons. Primers and fluorescent probes were designed with the LightCycler ProbeDesign program (v.2.0) and synthesized (Biomol, UK). The PCR reactions were performed with 50–100 ng genomic DNA, 0.5 μM of each primer, and 0.1 μM of each probe. PCR conditions were 95 °C, 2 min followed by 40 cycles (95 °C, 5 s; *X*°C, 15 s; 72 °C, 20 s). Annealing temperature (*X*) was 62 °C for rs5952, 60 °C for rs1467282, and a touchdown from 57 to 62 °C for rs1568565. Thermal conditions for the analysis of melting curves were 95 °C, 30 s followed by a gradient of 50–90 °C at a rate of 0.1 °C/s.

TBARS assay

To measure the levels of lipid peroxidation in the tissues, the 2-thiobarbituric acid (TBA) reactive species (TBARS) assay was performed. Tumor and normal colonic tissues were homogenized in PBS in the presence of butylated hydroxytoluene. Extracts were incubated with 0.2 M glycine–HCl, pH3.6, and TBA reagent (0.5 % TBA, 0.5 % SDS). After 15 min incubation at 90 °C, samples were cooled on ice and transferred to a 96-well microplate for triplicate readings. Absorbance was monitored at 532 nm in a Versamax microplate reader (Molecular Devices, USA). The experimental values were normalized to protein concentration. Two independent experiments with measurements in triplicate were performed.

Cell culture and immunocytofluorescence

The cell line HT-29 (kindly donated by Dr. C. Villalobos, IBGM, Spain) was cultured and maintained in Dulbecco's modified Eagle's medium (Lonza Iberica, Spain) with 10 % fetal bovine serum (FBS; Lonza Iberica), 1 % L-glutamine, and 1 % antibiotic cocktail (penicillin, 10 U/μl; streptomycin, 10 μg/μl; amphotericin B, 25 μg/ml; Lonza Iberica) at 37 °C in 5 % CO_2 with 90–95 % humidity.

To analyze the effect of 2′-deoxy-5-azacytidine (DAC; Sigma), this demethylating agent was added at 1 μM to the culture medium, and the cells were cultured for 24 h, as suggested by Hagemann et al. [19].

Paraquat (1,1′-dimethyl-4,4'-bipyridinium; Sigma) was added to the culture medium in the absence of FBS for either 6 h (to assess ApoD expression profile) or for 24 h (to evaluate cell death and proliferation).

Human ApoD purified from cystic fluid was kindly donated by Dr. E. Rassart, (University of Québec at Montreal, Canada) and added (4 nM) to the cell culture medium for 24 h in the absence of FBS.

Cells used for proliferation and apoptosis assays, as well as for ApoD immunofluorescence, were cultured onto poly-L-lysine (Sigma) coated coverslips. Fixation was performed in 4 % formaldehyde in PBS for 15 min, followed by washes in PBS, and cells were blocked and permeabilized in PBS with 0.25 % Triton X-100 and 1 % normal goat serum. A rabbit primary antibody antihuman ApoD (generated by C. López-Otín, Univ. de Oviedo) and an Alexa 488-conjugated secondary antibody were used for ApoD immunofluorescence. After mounting with Vectashield-DAPI (Vector Labs, USA), the cells were observed with an Eclipse 90i (Nikon) microscope, and images were taken with a DS-Ri1 (Nikon) digital camera, acquired with NIS-Elements BR 3.0 software (Nikon) and processed with ImageJ (v1.45 s). For normal, proliferating or apoptotic cell counting, we acquired images under the same conditions of illumination, diaphragm and condenser adjustments, exposure time, background correction, and color levels. A minimum of five ×20 fields randomly taken were used for quantification.

Proliferation and apoptosis assays

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to evaluate the DNA fragmentation that occurs in the last phase of apoptotic cell death. A TUNEL labeling kit (Roche) was used. Cell proliferation was assessed with the Clik-iT® EdU kit (Invitrogen) according to the manufacturer's protocol. This assay detects the newly synthesized DNA by incorporation of a modified nucleoside, 5-ethynyl-2′-deoxyuridine (EdU) during DNA synthesis.

Statistical analysis

Statistical analyses used for testing differences in expression levels were performed with Statgraphics plus (v 5.0) and Sigmaplot (11.0) softwares. $p<0.05$ was defined as a threshold for significant changes. SNPs association analysis was performed with SNPStats [20]; http://bioinfo.iconcologia.net/snpstats/start.htm].

Results

Lipid peroxidation increases with the clinical stage of colorectal cancer

The levels of lipid peroxidation adducts were evaluated with the TBARS method in our samples of adenocarcinoma and control adjacent tissue. Overall, the lipid peroxidation levels are increased in cancer tissues compared to normal mucosa

(Fig. 1a), as was previously described in blood [21]. However, when different stages of cancer development are taken into account, a maximum increase in TBARS signal was observed in stage IV adenocarcinomas (Fig. 1b).

ApoD expression levels in CRC progression

Many reports document low levels of ApoD in different forms of cancer [22–29]. In primary colorectal cancer, Ogawa et al. [30] corroborated a similar downregulation of ApoD messenger RNA (mRNA), and by establishing two categories of mRNA expression, they found a positive correlation between ApoD levels and CRC patient survival. However, an account of the development of ApoD expression in different tumor stages has not been studied. We therefore have examined the expression of ApoD in CRC tissue compared to normal colonic mucosa and have quantified ApoD gene expression by qRT-PCR and protein levels by immunoblot at different stages.

ApoD mRNA levels show an overall decrease in malignant tissue, with a particularly strong decline in mRNA levels in stage I CRC tissue (Fig. 2a). This sharp downregulation of mRNA in the initial stages of the tumor accounts for the decrease in protein levels observed in immunoblot studies (Fig. 2b). The reduced protein levels are then maintained during subsequent tumor developmental stages, with a lesser amount of downregulation in stages II and III.

Immunohistochemical localization of ApoD in normal mucosa and CRC tissue

ApoD is found to be expressed in cells of normal colonic mucosa both in the epithelium (white arrow in Fig. 3a) and the lamina propia (arrow in Fig. 3a). In the epithelium, ApoD labeling is observed in the cytoplasm of basally located cells (white arrow in Fig. 3b), with position and morphology resembling enteroendocrine cells (see "Discussion"). In the lamina propia, ApoD is seen as a cytoplasmic vesicular labeling in plasma cells (Fig. 3c, d), possibly in fibroblasts, a cell type known to express ApoD [31, 32]. Other unidentified ApoD-expressing cells are seen in the submucosa (arrow in Fig. 4e).

After studying the localization of cells expressing ApoD in the normal mucosa, we proceeded to find out whether the decrease in ApoD mRNA and protein levels observed in CRC malignant tissues (Fig. 2) is due to a general reduction in expression in the cells that normally express ApoD or alternatively to a heterogeneous decrease in ApoD-expressing cells (Fig. 4a–e). A general decrease in ApoD labeling is clear in the tumor tissue (Fig. 4a) compared to the normal tissue (Fig. 4e). However, the decrease in stromal labeling seen in malignant tissues when compared to the normal mucosa (white arrows in Fig. 4a, b vs. e), is commonly accompanied by an increase in the number of cells expressing ApoD in the dysplastic epithelium of the tumor tissue (arrows in Fig. 4c, d). Thus, regulation of ApoD protein expression in tumor tissues is more complex than a pure downregulation of ApoD.

Finally, we evaluated semiquantitatively the expression of ApoD in histological samples of tumor and normal mucosa tissues. The percentage of ApoD-expressing cells was estimated by scoring the staining obtained with immunohistochemistry. Net amounts of ApoD significantly decrease in malignant tissue progressively with CRC developmental stages (Fig. 4f).

Expression profile of ROS-dependent genes in CRC progression and coregulation with ApoD

The transcriptional levels of a set of 84 genes known to be related to ROS metabolism and antioxidant response were

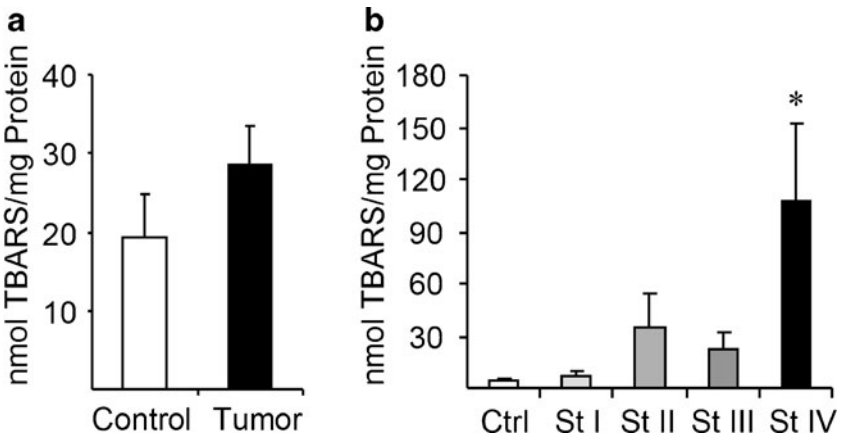

Fig. 1 Lipid peroxidation increases in terminal stages of colorectal cancer. **a** The levels of lipid peroxidation adducts, assayed by TBARS, increase in colorectal tumor tissue of pooled stages compared to control healthy mucosa tissue. **b** When analyzed independently in developmental stages of cancer progression the levels of lipid peroxidation increases mostly in stage IV. Data represent mean±SD of samples. Statistical differences were assayed by ANOVA test. *$p<0.05$

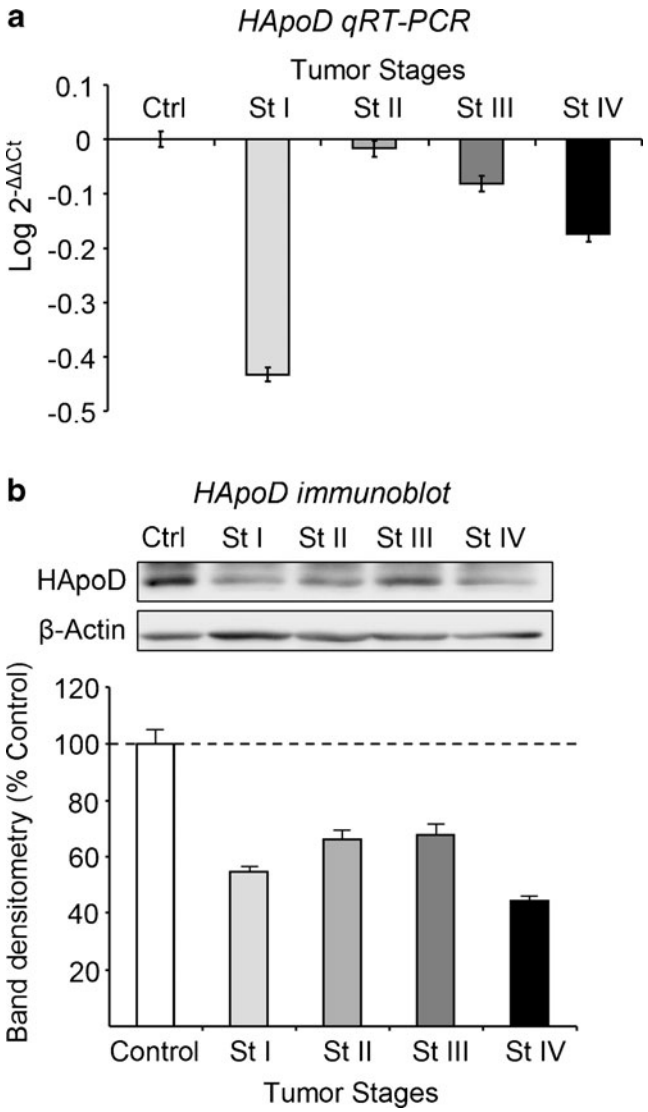

Fig. 2 Human ApoD is down-regulated in CRC tissues. **a** HApoD mRNA expression levels are decreased in colorectal tumor tissue, especially in stage I. Quantitative RT-PCR values are represented with respect to control conditions (calibrator sample). Relative amounts with respect to control healthy tissue in each tumor stage are shown. **b** HApoD protein levels remain low in tumor tissue during all stages of disease. Detection and quantification of HApoD was performed by immunoblot analysis. The blot shows a representative experiment of pooled samples for each stage with equal amounts of total protein extracts from control and CRC tissues. *Bar graph* represents mean± SD of three independent immunoblot experiments. Densitometry values were normalized to β-actin and shown as percent of control values. Statistical differences assayed by Mann–Whitney *U* test in **a** and by ANOVA test in **b**. *$p<0.05$

evaluated by qRT-PCR using the GAPDH gene as a ubiquitous control and the levels of gene transcription in normal tissues as the calibrator for relative quantification.

Most of the genes of our array showed an upregulation in the tumor tissue as compared to normal colonic tissue (Fig. 5a), and 32 genes showed significant changes of expression in the tumor tissues according to the criteria described in "Materials and methods." The complete list of genes showing significant changes is in Table 2.

Since we have studied the gene expression profile in different stages (I–II, III, and IV), we were interested in looking at the patterns of gene expression with tumor progression. Most of the genes studied (63 %) showed a peak of expression at stage III (expression pattern type 1; Fig. 5b), while 13 % show higher expression at stages I–II (expression pattern type 2; Fig. 5c). Some genes, like SGK2, show an upregulation in all of the CRC stages (expression pattern type 3; Fig. 5c), while only 6 % of genes show increased expression with advanced stages of tumor development (expression pattern type 4; Fig. 5e).

The expression profile study also allows searching for genes that show coregulation of expression with ApoD, as possible targets to study ApoD-related gene pathways. The genes showing downregulation with tumor development account for 7 % of the total studied (expression pattern type 5; Fig. 5f), and some of them are functionally linked in a gene network (Fig. 1S and Table 1S).

ApoD polymorphisms and CRC risk

Several SNPs of the human ApoD gene have been reported to associate with disease states such as Alzheimer's (AD) and schizophrenia [33, 34]. We have genotyped three SNPs (rs5952, rs1568565, and rs1467282) that have been associated to AD [33, 35]. The single and multiple SNPs analyses are shown in Tables 2S and 3S. A study of the haplotypes generated by the SNPs analyzed in our study found no significant association with CRC risk (Table 3S).

ApoD expression in the CRC cell line HT-29 and its role in proliferation and cell death

DNA methylation is a mechanism by which tumor cells silence gene expression [36], and several reports have shown that ApoD is a hypermethylated cancer-associated gene [29, 30, 37]. ApoD is upregulated by, and protects against, oxidative stress [9, 13, 15]. As reported here and in other works, ApoD expression is maintained at general low levels in tumor tissues, which, along with its positive regulation by growth arrest [38], supports the proposal made by several authors that ApoD is a tumor suppressor gene [30]. Nevertheless, malignant cells generate high levels of ROS and boost lipid peroxides, which in turn should increase ApoD expression [16]. How could we explain this paradox?

We set up to test the effect of ApoD silencing by methylation and ApoD regulation by stress, on the proliferation and death of the CRC cell line HT-29 (Fig. 6). Demethylation with DAC liberates ApoD from methylation-induced silencing and increases its expression (1.6-fold mRNA

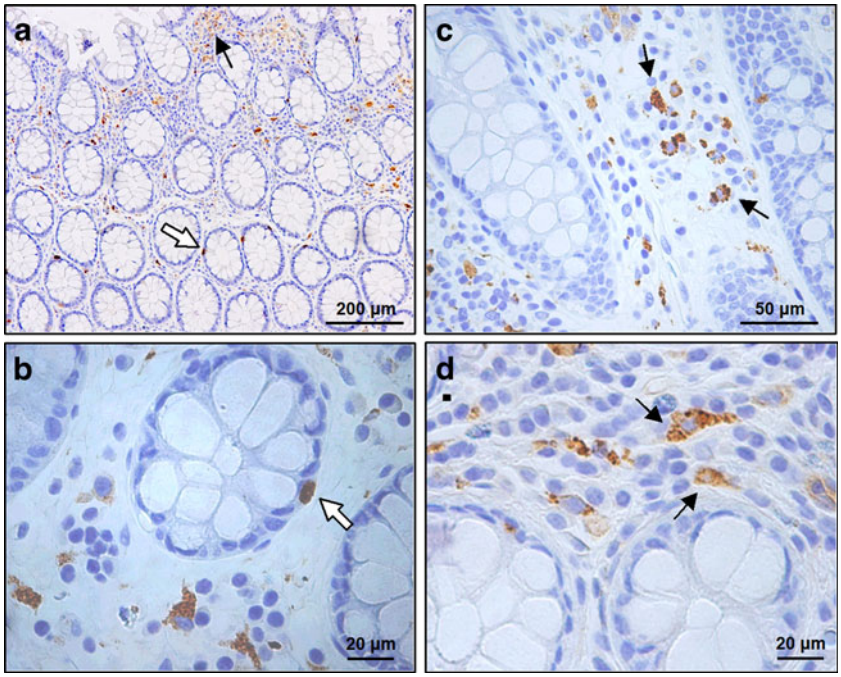

Fig. 3 ApoD protein expression in normal colon tissue. Mesenchymal cells express ApoD in normal mucosa (*arrows* in **a**, **c**, and **d**) and a small number of possibly enteroendocrine cells show ApoD labeling in the colonic crypts (*white arrows* in **a** and **b**)

induction; Fig. 6a) to levels known to significantly decrease cell growth [29]. PQ treatment increases ApoD expression to levels similar to those attained by treatment with DAC. Our analysis also suggests that there is an upper limit of endogenous expression that can be reached either by demethylation or after exposure to OS, as no further ApoD expression is attained by simultaneous treatment with DAC and PQ (Fig. 6a). Curiously, these constantly proliferating cells are able to express ApoD while they have not entered cell cycle. This is clear in Fig. 6b, where only EdU-negative cells (arrows) express ApoD.

Demethylation with DAC, which causes an increase in ApoD expression, affects neither cell proliferation (Fig. 6c) nor cell death (Fig. 6d) under control culture conditions. On the other hand, serum starvation stress halts proliferation as expected (Fig. 6c) without significant effects on cell death (Fig. 6d). We then tested whether the exogenous addition of human ApoD to the cell culture medium affects cell growth. In control conditions, ApoD added to the culture medium at concentrations known to protect cells from stress [16] does not have a significant effect in proliferation or apoptosis levels (Fig. 6c,d).

As mentioned above, tumor growth increases ROS and inflammation levels. The treatment of HT-29 cells with the ROS generator paraquat (PQ), which induces ApoD expression in these cells (Fig. 6a) and in a number of experimental models [13, 15, 16], shows a dramatic effect on both cell proliferation and cell death (Fig. 6c, d). Interestingly, the simultaneous addition of exogenous ApoD does not affect proliferation (Fig. 6b), but significantly increases apoptotic cell death upon PQ-induced oxidative stress (Fig. 6c).

Discussion

Oxidation state of CRC along stages and its relationship to ApoD expression

The particular metabolic demands of cancer cells are known to generate increased levels of ROS in many tumor types, and this in turn results in peroxidation of cell membrane phospholipids. An increase in lipid peroxidation, both in blood and cancerous tissue samples, has been previously reported in primary CRC patients [39–41]. In particular, Skrzydlewska et al. [41] found a steady increase of the lipid peroxide adduct MDA in malignant tissue of stages II–IV and a sustained increase in 4-HNE. Although our results confirm the previous findings, we observed a sharp increase in lipid peroxide adducts in stage IV of CRC. Our results are also in agreement with the reported deployment in stage IV CRC of nonenzymatic antioxidants such as GSH and vitamins C and E [41].

An extensive literature supports the notion that tumor cells maintain low levels of ApoD in their surroundings, and a decreased mRNA and/or protein expression has been reported in breast [22], ovary [23], prostate [24], hepatic [25, 26], neural [27, 28], esophageal cancer [29], and colorectal cancer [30]. Our data show a prominent decrease in mRNA and protein levels in the initial stages of CRC. While mRNA levels are kept below normal throughout the progression of the tumor, they increase with respect to the stage I levels, in parallel with the increase in lipid peroxidation adducts, indicating a complex temporal regulation of ApoD depending on the phisiological state of the tissue.

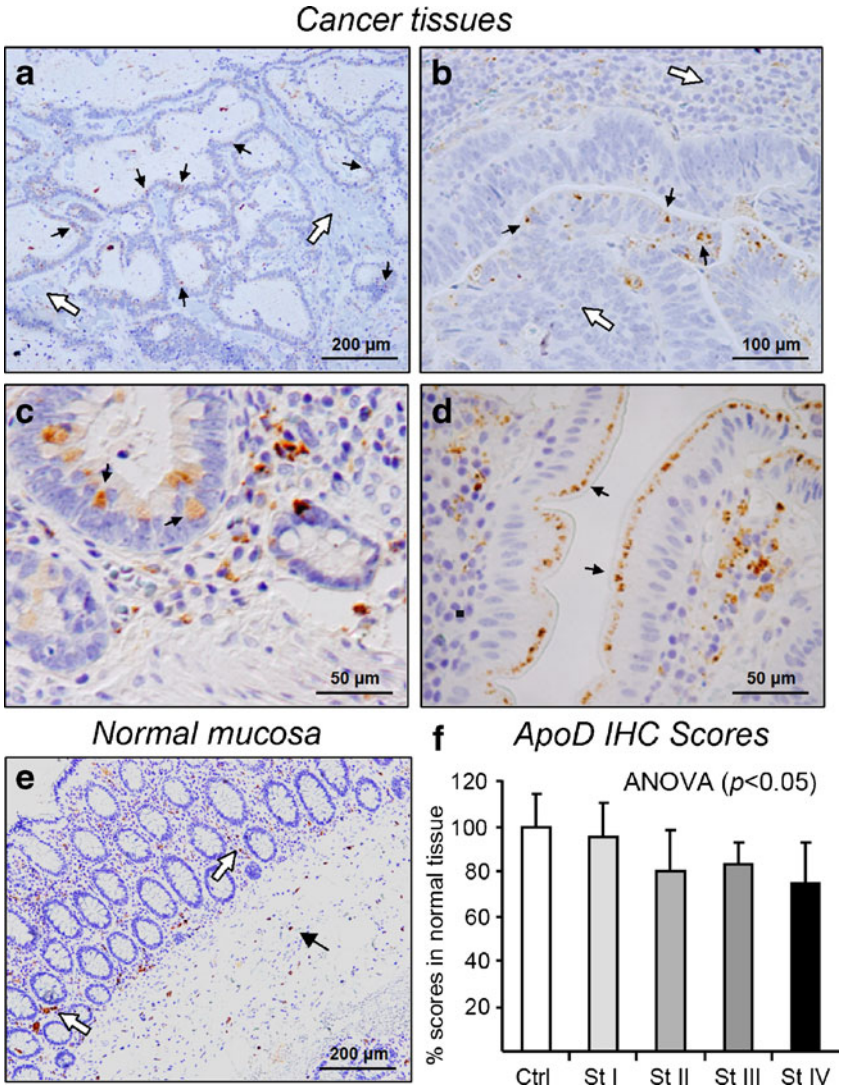

Fig. 4 Human ApoD is globally down-regulated in colorectal tumors but shows heterogeneous changes of expression in different cell types. **a**–**d** HApoD protein expression in CRC tissues. *White arrows* point to stromal tissue that shows minimal ApoD labeling. *Arrows* indicate ApoD-positive cells in the dysplastic epithelium and carcinoma tissue. **e** ApoD expression in normal mucosa shown for comparison. **e** Semiquantitative expression of ApoD by scoring immunohystochemical labeling of samples according to their developmental tumor stage. Data represent mean±SD of samples. Statistical differences were assayed by Anova test. *$p<0.05$

Cell-type distribution of ApoD expression in normal and cancerous colorectal tissues

As shown above, in addition of the ApoD-expressing cells in the lamina propia, ApoD-positive cells are observed in the epithelium. These cells, according to their location and morphology, could be the endocrine cells of the crypts, known to be secreting a number of hormones as well as cell stress proteins such as heat shock protein 1 and superoxide dismutase 1 [42]. Interestingly, enteroendocrine colonic cells are involved in immune activation and inflammatory response (reviewed by [43]), a role that has also been proposed for ApoD [16, 44, 45]. In CRC, ApoD immunoreactivity changes heterogeneously, with more ApoD-positive cells generally seen in the dysplastic epithelium. These altered epithelial cells might be those keeping their ability to respond to the increasing oxidative stress by upregulating ApoD. The remaining malignant cells downregulate ApoD expression, thus resulting in a global decrease in ApoD. We propose that a balance between two different cellular behaviors can be the final determinant of the patient outcome. Understanding this differential regulation of ApoD along the different stages of CRC is therefore of special interest for further research.

The transcriptional responses of ROS-dependent genes parallel or oppose ApoD expression changes throughout the stages of CRC

We observe a general upregulation of antioxidant gene expression when comparing CRC and control tissues, as it has been previously described [41, 46]. It accounts for the timely cell response to cope with the increasing levels of oxidative stress generated by the abnormal proliferation of cancerous cells. However, the different patterns of expression along CRC stages reveal subsets of genes functionally linked that deserve more studies. Only a few of the genes

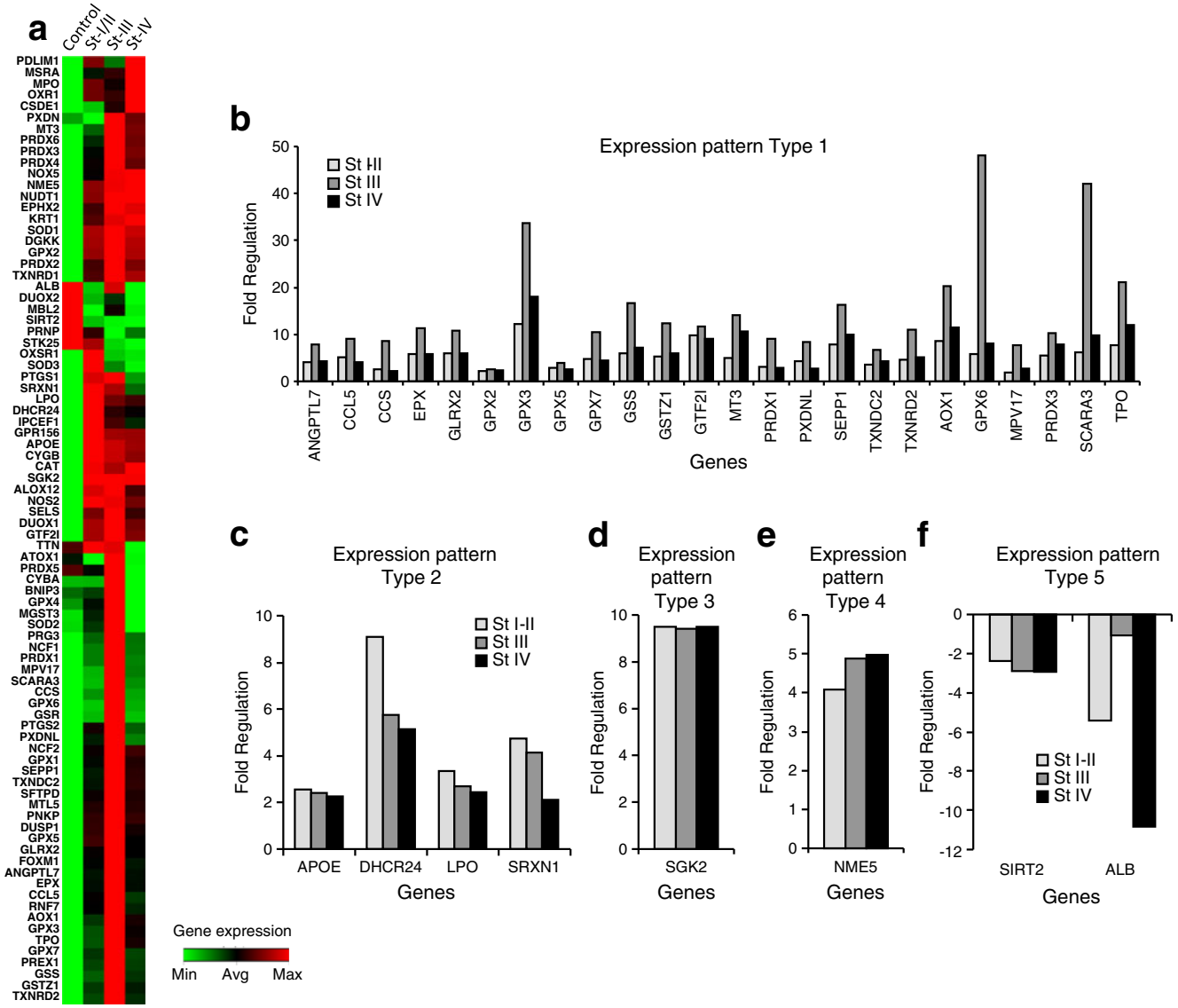

Fig. 5 Quantitative RT-PCR expression profiles of OS-related genes in CRC tissues of different developmental stages. **a** Cluster analysis and heat map of the genes that showed genotype-dependent significant differences. *Columns* represent samples, and *rows* represent genes. Relative quantification scaling and corresponding color codes are shown at the *bottom-left*. Fold change values are listed in Table 2. **b**–**f** Different patterns of gene expression are shown

studied respond, like ApoD, with a decrease in expression. Moreover, though not explored in our arrays, the gene PACAP was also found strongly downregulated in CRC tissues [47], and it is known to participate in the regulation of ApoD expression [48].

Another interesting finding of our study is the different expression pattern exhibited by ApoE (expression pattern type 2; upregulated) and ApoD (expression pattern type 5; downregulated), two proteins frequently proposed to show coregulation because of their presumed function in lipoprotein metabolism. These opposite expression profiles, already reported in our previous studies on ApoD function in the nervous system [16, 45], point to different roles for the two lipoproteins.

It is interesting to note that despite a consistent downregulation has been found in many cancer types for ApoD, this gene does not stand as a CRC biomarker in several metanalyses of expression profiles [49, 50]. We think that these results are due to the expression changes with tumor progression reported above. Instead, and based on our results, ApoD could be used as a marker for initial stages of tumor invasion beyond the colonic mucosa.

ApoD as a cell-death-promoting factor in CRC cells

In this work, we verify that the ApoD promoter is basally methylated in the CRC HT-29 cell line, which silences to

Table 2 Expression profiles of genes related to ROS metabolism and antioxidant response in CRC tumor tissues of different stages

	Up–down regulation (comparing to control group)									
	Stages I and II		Stage III		Stage IV		*p* value (comparing to control group)			
	Fold regulation	Comments	Fold regulation	Comments	Fold regulation	Comments	Group 1	Group 2	Group 3	Gene name
ALB	−5.42		−1.08		−10.83		0.000359	0.344061	0.000179	Albumin
ALOX12	5.33	A	5.64	A	3.93	A	0.014894	0.000001	0.000021	Arachidonate 12-lipoxygenase
ANGPTL7	4.18		7.85		4.23		0.001781	0.001177	0.022402	Angiopoietin-like 7
AOX1	8.66		20.24	A	11.55		0.005527	0.000167	0.000106	Albumin
APOE	2.56		2.40		2.26		0.012312	0.000031	0.000003	Apolipoprotein E
ATOX1	−1.64	B	1.45	B	−1.62	B	0.207943	0.2676	0.204858	ATX1 antioxidant protein 1 homolog (yeast)
BNIP3	1.13		2.10		−1.82		0.355284	0.000285	0.004312	BCL2/adenovirus E1B 19 kDa interacting protein 3
CAT	5.79	A	5.08	A	5.90	A	0.020306	0.000017	0.000092	Catalase
CCL5	5.10		9.08		4.15		0.003117	0.000887	0.00001	Chemokine (C–C motif) ligand 5
CCS	2.58		8.67		2.32		0.006973	0.000001	0.000069	Copper chaperone for superoxide dismutase
CSDE1	1.07	B	1.37	B	1.65		0.750389	0.164293	0.020505	Cold shock domain containing E1, RNA-binding
CYBA	1.01		3.26		−1.53		0.816368	0.000371	0.000681	Cytochrome b-245, alpha polypeptide
CYGB	2.86	A	2.67	A	2.45	A	0.007389	0.000237	0.000035	Cytoglobin
DGKK	12.50	A	14.98	A	13.00	A	0.00185	0.000002	0.000013	Diacylglycerol kinase, kappa
DHCR24	9.11		5.75		5.14		0.000015	0.000051	0.000469	24-dehydrocholesterol reductase
DUOX1	10.94	A	12.97	A	9.61	A	0.003242	0.000002	0.000016	Dual oxidase 1
DUOX2	−1.61	A	−1.35		−1.79		0.016687	0,014672	0,001593	Dual oxidase 2
DUSP1	13.98	A	22.63	A	12.67	A	0.004939	0.000001	0.000186	Dual specificity phosphatase 1
EPHX2	4.21	A	6.15	A	5.88		0.010745	0.00022	0.000494	Epoxide hydrolase 2, cytoplasmic
EPX	5.91		11.27		5.88		0.003998	0.000002	0.00003	Eosinophil peroxidase
FOXM1	3.53	A	6.15	A	3.36	A	0.005489	0.000023	0.000036	Forkhead box M1
GLRX2	6.05		10.90		5.94		0.000987	0.000155	0.000051	Glutaredoxin 2
GPR156	3.97	A	3.42	A	3.38	A	0.005431	0.000054	0.000003	G protein-coupled receptor 156
GPX1	18.39	A	36.88	A	21.39	A	0.005394	0.000028	0.000002	Glutathione peroxidase 1
GPX2	2.32		2.65		2.39		0.01818	0.000054	0.000088	Glutathione peroxidase 2 (gastrointestinal)
GPX3	12.21		33.67		18.00		0.002722	0.000003	0.000001	Glutathione peroxidase 3 (plasma)
GPX4	1.52	B	2.54		−1.63		0.050446	0.00028	0.019233	Glutathione peroxidase 4 (phospholipid hydroperoxidase)
GPX5	2.85		3.99		2.50		0.010091	0.006701	0.000062	Glutathione peroxidase 5 (epididymal androgen-related protein)
GPX6	5.87		48.16	A	8.07		0.017343	0.000026	0.003525	Glutathione peroxidase 6 (olfactory)
GPX7	4.80		10.55		4.48		0.004305	0	0.000825	Glutathione peroxidase 7
GSR	2.01	B	8.67		1.90		0.068594	0.000002	0.030164	Glutathione reductase

Table 2 (continued)

	Up–down regulation (comparing to control group)									
	Stages I and II		Stage III		Stage IV		*p* value (comparing to control group)			
	Fold regulation	Comments	Fold regulation	Comments	Fold regulation	Comments	Group 1	Group 2	Group 3	Gene name
GSS	5.95		16.66		7.29		0.00315	0	0.001624	Glutathione synthetase
GSTZ1	5.41		12.29		6.07		0.00805	0.000019	0.000012	Glutathione transferase zeta 1
GTF2I	9.85		11.70		9.09		0.001747	0	0	General transcription factor II, i
KRT1	11.90	A	16.67	A	17.51	A	0.001463	0.000019	0.000035	Keratin 1
LPO	3.35		2.69		2.46		0.010494	0.000057	0.000225	Lactoperoxidase
MBL2	−1.81		−1.26		−1.77	A	0.01184	0.013227	0.00087	Mannose-binding lectin (protein C) 2, soluble (opsonic defect)
MGST3	1.44	A	2.07	A	−1.06	B	0.140885	0.00217	0.63463	Microsomal glutathione S-transferase 3
MPO	13.98	A	10.65	A	19.00	A	0.001465	0.00001	0.000002	Myeloperoxidase
MPV17	1.96	B	7.66		2.69		0.110412	0.000008	0.000452	MpV17 mitochondrial inner membrane protein
MSRA	153.76	A	199.06	A	332.55	A	0.002594	0	0.000004	Methionine sulfoxide reductase A
MT3	5.06		14.01		10.58		0.008775	0	0	Metallothionein 3
MTL5	5.44	A	8.80	A	5.48		0.003815	0	0.000235	Metallothionein-like 5, testis-specific (tesmin)
NCF1	1.52		3.08		1.55		0.021764	0.000091	0.002019	Neutrophil cytosolic factor 1
NCF2	19.01	A	35.69	A	22.58	A	0.002443	0.000002	0.000055	Neutrophil cytosolic factor 2
NME5	4.08		4.87		4.97		0.00193	0.00003	0.00007	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)
NOS2	1.31		1.30		1.22		0.19083	0.00959	0.03356	Nitric oxide synthase 2, inducible
NOX5	3.04	A	4.91	A	4.97	A	0.01021	0.00001	0.00000	NADPH oxidase, EF-hand calcium binding domain 5
NUDT1	12.39	A	15.83	A	15.88	A	0.00175	0.00000	0.00000	Nudix (nucleoside diphosphate linked moiety X)-type motif 1
OXR1	208.45	A	175.64	A	290.40	A	0.00177	0.00000	0.00000	Oxidation resistance 1
OXSR1	43.45	A	5.49		2.88		0.00233	0.00030	0.00001	Oxidative-stress responsive 1
PDLIM1	1.46		1.17		1.61		0,02587	0.13411	0.00386	PDZ and LIM domain 1
IPCEF1	1.16		1.10		1.07		0.33359	0.00689	0.08616	Interaction protein for cytohesin exchange factors 1
PNKP	9.07	A	14.59	A	9.21		0.00131	0.00000	0.00002	Polynucleotide kinase 3'-phosphatase
PRDX1	3.07		9.15		2.95		0.01417	0.00001	0.00025	Peroxiredoxin 1
PRDX2	2.83	A	3.89	A	3.15	A	0.00774	0.00001	0.00000	Peroxiredoxin 2
PRDX3	5.56		10.30	A	7.88		0.00323	0.00000	0.00010	Peroxiredoxin 3
PRDX4	12.91	A	23.91	A	16.93	A	0.00285	0.00001	0.00000	Peroxiredoxin 4
PRDX5	−1.10		1.17		−1.50		0.58461	0.11277	0.00703	Peroxiredoxin 5
PRDX6	8.42	A	18.66	A	13.66		0.00130	0.00039	0.00013	Peroxiredoxin 6
PREX1	11.07	A	28.36	A	11.36	A	0.00280	0.00007	0.00005	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1

Table 2 (continued)

	Up–down regulation (comparing to control group)									
	Stages I and II		Stage III		Stage IV		*p* value (comparing to control group)			
	Fold regulation	Comments	Fold regulation	Comments	Fold regulation	Comments	Group 1	Group 2	Group 3	Gene name
PRG3	1.95	B	4.11		1.85		0.06409	0.00017	0.00936	Proteoglycan 3
PRNP	−1.10		−1.30		−1.20		0.55793	0.01056	0.01490	Prion protein
PTGS1	2.37		2.47	B	1.29	B	0.02002	0.13761	0. 22171	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
PTGS2	3.24		5.13		2.31	B	0.00584	0.00059	0.05500	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
PXDN	−1.09		1.37		1.24		0.60194	0.00243	0.00023	Peroxidasin homolog (Drosophila)
PXDNL	4.34		8.48		2.82		0.01486	0.00003	0.,00244	Peroxidasin homolog (Drosophila)-like
RNF7	3.66	A	6.31	A	3.29	A	0.01086	0.00001	0.00001	Ring finger protein 7
SCARA3	6.26		42.02	A	9.71		0.00322	0.00011	0.01597	Scavenger receptor class A, member 3
SELS	5.74	A	7.47	A	4.95	A	0.00530	0.00003	0.00011	Selenoprotein S
SEPP1	7.90		16.38		9.98		0.00099	0.00012	0.00165	Selenoprotein P, plasma, 1
SFTPD	5.26	A	9.13	A	5.53		0.00230	0.00033	0.00031	Surfactant protein D
SGK2	9.52		9.52		9.41		0.00218	0.00019	0.00000	Serum/glucocorticoid regulated kinase 2
SIRT2	−2.40		−2.88		−2.96		0.00060	0.00000	0.00000	Sirtuin (silent mating type information regulation 2 homolog) 2 (S. cerevisiae)
SOD1	8.26	A	9.71	A	8.81	A	0.00572	0.00000	0.00000	Superoxide dismutase 1, soluble
SOD2	1.25		1.71		−1.06		0.28408	0.00787	0.62557	Superoxide dismutase 2, mitochondrial
SOD3	77.56	A	18.80	A	2.14		0.00274	0.00000	0.00138	Superoxide dismutase 3, extracellular
SRXN1	4.76		4.13		2.11		0.00401	0.00001	0.00016	Sulfiredoxin 1 homolog (S. cerevisiae)
STK25	−1.09		−1.78		−1.89		0.70550	0.00239	0.00272	Serine/threonine kinase 25 (STE20 homolog, yeast)
TPO	7.72		21.16	A	11.94		0.00761	0.00001	0.00001	Thyroid peroxidase
TTN	1.19		1.16		−1.53		0.29119	0.01195	0.00120	Titin
TXNDC2	3.66		6.74		4.38		0.00080	0.00001	0.00201	Thioredoxin domain containing 2 (spermatozoa)
TXNRD1	16.92	A	25.34	A	21.14	A	0.00482	0.00000	0.00000	Thioredoxin reductase 1
TXNRD2	4.55		10.94		5.15		0.00617	0.00005	0.00004	Thioredoxin reductase 2

GAPDH gene was used as a ubiquitous control, and the level of gene transcription in normal tissues was the calibrator for relative quantification. Fold change ($2^{-\Delta\Delta Ct}$) is the normalized gene expression ($2^{-\Delta\Delta Ct}$) in the test Sample divided by the normalized gene expression ($2^{-\Delta Ct}$) in the control sample. Fold regulation represents fold-change results in a biologically meaningful way. Fold-change values > 1 indicate a positive or upregulation, and the fold regulation is equal to the fold change. Fold-change values < 1 indicate a negative or downregulation, and the fold regulation is the negative inverse of the fold change. The *p* values are calculated based on a Student's *t* test of the replicate $2^{-\Delta Ct}$ values for each gene in the control group and treatment groups

A the gene expression is relatively low in one sample (Ct > 30) and reasonably detected in the other sample (Ct < 30), suggesting that the actual fold-change value is at least as large as the calculated fold-change result; *B* the gene average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the *p* value for the fold change is either unavailable or relatively high ($p > 0.05$)

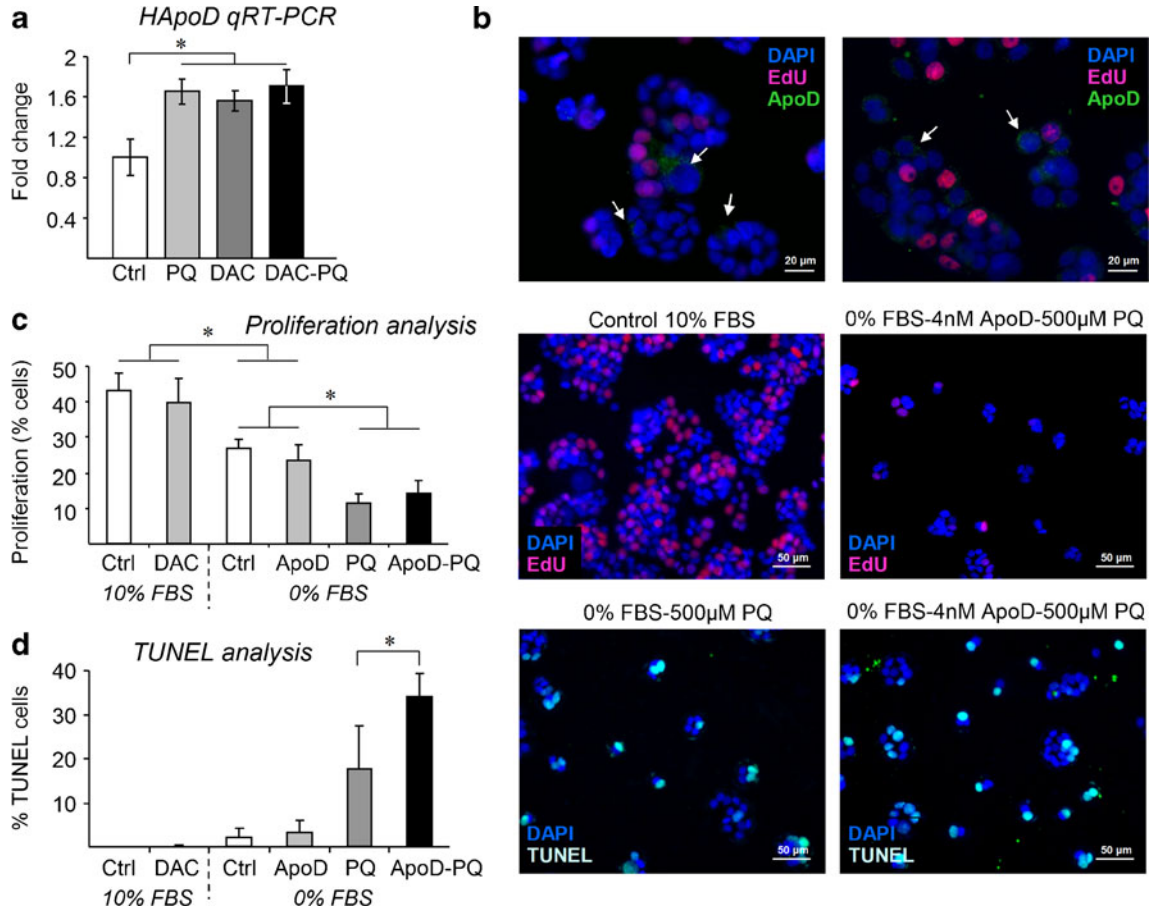

Fig. 6 Cell proliferation and apoptotic death levels of HT-29 CRC cells in relation to ApoD expression and OS levels. **a** Quantitative RT-PCR expression of ApoD in HT-29 CRC cultures subjected to oxidative stress (*PQ*), demethylation (*DAC*), or both treatments, represented with respect to control conditions (calibrator sample). Statistical differences were assayed by Mann–Whitney *U* test; **p*<0.05. **b** Immunofluorescent pictures showing endogenous ApoD expression (*white arrows*) only by cells that have not entered the cell cycle. **c**, **d** Cell proliferation (**c**) and apoptotic cell death (**d**) analyses under different conditions of serum concentration, demethylating treatment (*DAC*), experimental oxidative stress (*PQ*), and/or addition of exogenous ApoD. Representative immunofluorescence pictures for each assay are shown on the *right*

some extent the expression of this lipocalin while the cells are undergoing cell division. However, HT-29 cells do indeed express ApoD, and they retain the ability to overexpress this protein in response to escalating OS. We have previously shown in PQ-challenged astrocyte primary cultures that the concentration of ApoD is critical for its protective effect [16]. An excess of ApoD appears to be detrimental for cell survival, a result also supported by the fact that ApoD gene transcription is transient in response to OS [13, 16].

Our data show that the situation in the colorectal tissue would in fact be more complex. Stromal cells would silence ApoD expression, perhaps by methylating the ApoD gene, while cancer cells could still express ApoD when they are not dividing (Fig. 6). The general decrease in ApoD protein expression in the malignant tissue is accompanied by increased numbers of cells expressing ApoD in the dysplastic epithelium. When tumor growth progresses OS builds up in the tissue, cells that induce ApoD expression account at least in part for the recovery of expression after the initial down-regulation at early stages (Fig. 2). This effect might be a key factor determining the final levels of ApoD in the tissue, which might favor apoptosis of tumor cells and therefore could influence the final patient survival outcome [30].

Another apolipoprotein, ApoL6, is located in a chromosomal region recently found to be one of the genomic hotspots clinically associated to tumor aggressiveness [51]. Curiously, this apolipoprotein has also been demonstrated to be a proapoptotic factor [52].

Since no loss-of-function mutation in ApoD has been associated to cancer so far, ApoD cannot be classified as a classical tumor suppressor gene. However, our results strongly support that ApoD is one of the genes that respond to the anomalous physiology occurring in the cancer microenvironment. ApoD might be a beneficial protein for CRC not by controlling the proliferative state of malignant cells, but by

promoting their death in the pro-oxidant environment of the tumor. In this context, ApoD could be used as an exogenous tool to promote the death of proliferating tumor cells when they are simultaneously suffering oxidative stress.

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