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Effects of airborne particulate matter on alternative pre-mRNA splicing in colon cancer cells

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A B S T R A C T

Alternative pre-mRNA splicing plays key roles in determining tissue- and species-specific cell differentiation as well as in the onset of hereditary disease and cancer, being controlled by multiple post- and co-transcriptional regulatory mechanisms. We report here that airborne particulate matter, resulting from industrial pollution, inhibits expression and specifically affects alternative splicing at the 5′ untranslated region of the mRNA encoding the bone morphogenetic protein BMP4 in human colon cells in culture. These effects are consistent with a previously reported role for BMP4 in preventing colon cancer development, suggesting that ingestion of particulate matter could contribute to the onset of colon cell proliferation. We also show that the underlying mechanism might involve changes in transcriptional elongation. This is the first study to demonstrate that particulate matter causes non-pleiotropic changes in alternative splicing.

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1. Introduction

Airborne particulate matter (PM) has been linked to many adverse health effects, in particular on cardiorespiratory diseases (Araujo and Nel, 2009; Lipsett et al., 2011), type II diabetes (Meo et al., 2015) and cancer (Krewski et al., 2009; Raaschou-Nielsen et al., 2011; Loomis et al., 2013). Jeddah, the second largest city in Saudi Arabia, is a setting of high PM exposure. Components of the PM from Jeddah were characterized by Khoder et al. (2012). PM collected from Jeddah was found to increase expression of genes whose products belong to pathways associated with disease including metabolic syndrome and atherosclerosis both in vitro (Sun et al., 2012) and in vivo (Brocato et al. 2014). Recent studies revealed that the average concentrations of PM10 in Jeddah were clearly much higher than the limit established by the World Health Organization (WHO) of 50 μg/m3 for 24 hours (Alghamdi et al. 2015a, Khoder et al. 2012). The elemental composition of PM10 revealed that tey are rich in Cr, Mn, Sr, Co, As, Pb, Cd, Ni and V (Khoder et al., 2012, Alghamdi et al., 2015a). There are also data for one group of specific organic compounds, the polycyclic aromatic hydrocarbons in Jeddah (Alghamdi et al., 2015b). These do not comprise a large proportion of particulate matter mass, but are important due to the carcinogenicity of some members of this group of compounds.

Colorectal cancer (CRC) is a serious life-threatening health problem in Saudi Arabia. It is the number one cancer in the male Saudi population. CRC has been related to various dietary, life style and environmental factors. Among these, high consumption of red meat, low intake of folate, smoking, lack of activity, and exposure to environmental pollutants were found to play a significant role in colorectal carcinogenesis. Particulate air pollution is derived primarily from vehicular traffic and burning of fossil fuels, and it may also arise from the desert environment. Accordingly, elevated risks of colon cancer were found among petrol station/automobile repair workers, workers exposed to asbestos, soot, cutting fluids/oils and combustion gases from coal/coke/wood (De Verdier et al., 1992). Recently an increase in mortality due to CRC in towns lying in the vicinity of metal production and processing installations

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was reported (García-Pérez et al., 2010).

There is a misconception that the main regulatory event determining cell differentiation and functioning is the differential control of gene expression at the level of transcription. The role of alternative pre-mRNA splicing in the definition of cell behavior in response to environmental cues is greatly neglected. We know now that the regulation of gene expression not only implies the knowledge of which genes are turned on and which ones are turned off, but also implies the detailed knowledge of which alternative splicing isoforms are produced from those genes that are "on" (for a review see Kornblihtt et al., 2013). Therefore, the purpose of the present study is to determine whether airborne particulate matter has an effect on gene expression in colon cancer cells at the level of alternative splicing and whether this effect is of a general nature or specific to certain genes. We found that exposure of colon cancer cells in culture to PM10 specifically affects alternative splicing of the pre-mRNA for the bone morphogenetic protein BMP4. It does this by favoring a splicing isoform that does not alter the encoded amino acid sequence but that reduces the overall expression of the protein. This is consistent with previously reported evidence (Lombardo et al., 2011) that inhibition of BMP4 expression may contribute to the onset of colon cancer.

2. Materials and methods

2.1. Particle sample collection

Dust samples were collected from the campus of King Abdulaziz University, located in south Jeddah. Particles were collected for 48 h on 5300 Polypropylene filters using a Staplex high volume air sampler (Staplex Air Sampler Division, USA) with PM10 inlet (serial no. 2840) at a fixed flow rate of 900 l/min.

Particle extraction: particles of 10 μm diameter or less (PM10) were extracted from polypropylene filters using a modified aqueous extraction protocol (Duvall et al. 2008). Briefly, each filter was wetted with 25 ml of 70% ethanol followed by sonication in 100 ml of distilled water for 2 h. The particles were dried by lyophilization, then weighed and stored at −80 °C.

2.2. Cell culture and transfection

HEK293T (human embryonic kidney) and HCT116 (human colon cancer) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g of glucose and 10% fetal bovine serum (Gibco, Life Technologies, Gaithesburg, MD, USA) at 37 °C.

Cell viability was determined by trypan blue exclusion; cells were plated at a density of 5.10^4/well in 24-well plates and cultured for 48 h with the stated amount of PM10 particles. After incubation, the cells were released with trypsin/EDTA, incubated with trypan blue, and counted using a hemocytometer.

Camptothecin (Sigma-Aldrich, St. Louis, MO, USA) 6 μM was added 4 h before cell harvest. For siRNA experiments, cells were transfected with siLuc (5′CUUACGCUGAGUACUUCGA3′), at a final concentration of 20 nM. Approximately 48 h after transfection, RNA was extracted for RT-PCR studies.

2.3. RNA extraction and RT-PCR

Total RNA was extracted with Tri-Reagent (Ambion) according to the manufacturer's instructions. This method combines phenol and guanidine thiocyanate in a monophasic solution to rapidly inhibit RNase activity.

One microgram of RNA was reverse transcribed with the M-MLV reverse transcriptase (Invitrogen) and oligo-dT primer, and the cDNA was amplified with human BMP4 primers 5′

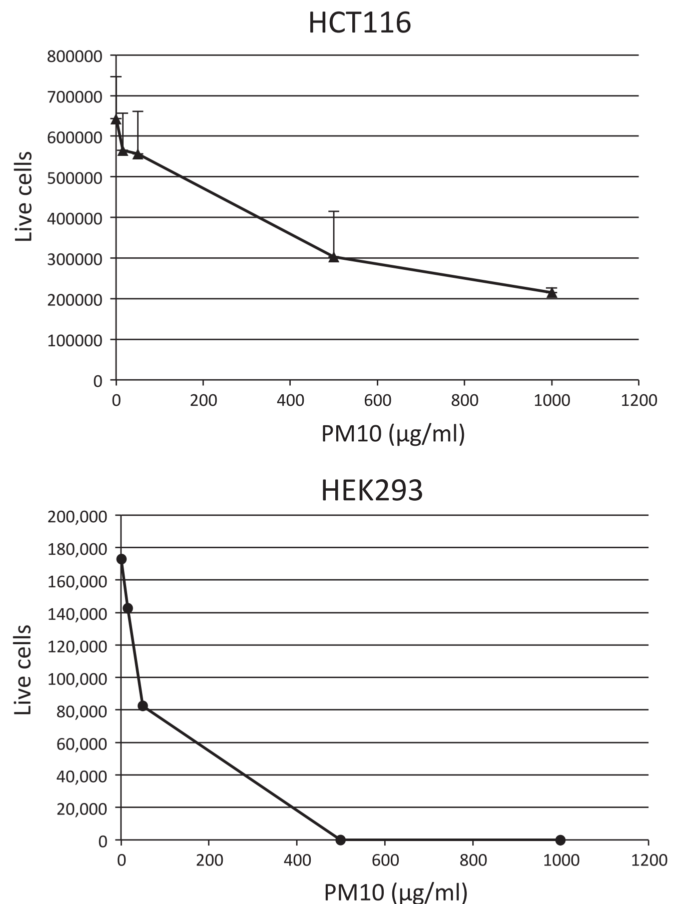

Fig. 1. Effect of PM10 particles on cell viability of the human colon carcinoma cell line HCT116 and the human embryo kidney transformed cell line HEK293. Cells were incubated with increasing amounts of PM10 for 48 h and counted as described in Section 2. HCT116 data correspond to average and SD of three independent experiments. HEK293 data correspond to a single representative experiment.

CGAGAAGGCAGAGGAGGAG3′ and 5′CAAACTTGCTGGAAAGGCTC3′. Radioactive PCR conditions and alternative splicing evaluation through native polyacrylamide gel electrophoresis were done as previously reported (de la Mata et al., 2003). To measure BMP4 mRNA levels through real time PCR, cDNA was amplified using primers 5′CACTGGTCTTGAGTATCC3′ and 5′CTGCTGAGGTTAAAGAGG3′ mapping to BMP4 exons 3 and 4 respectively. Amplification of the control mRNA of Hsp90 was carried out using primers 5′CCAAAAAGCACCTGGAGATCA3′ and 5′TGTCGGCCTCAGCCTTCT3′.

2.4. Pol II elongation measurement

An adaptation of the method developed by Singh and Padgett (2009) was used. A DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole) treatment of 300 mM for 5 h was necessary in order to fully block endogenous transcription of the human fibronectin gene (FN1). For the CPT-treated cells, CPT 1 mM was added 30 min before the DRB wash and remained until cell harvest. Total RNA was extracted as mentioned above. Reverse transcriptase reaction was initiated with random decamers. Quantification of the pre-mRNAs was performed on the fibronectin gene by real-time PCR with an amplicon spanning the exon 1/intron1 junction. The primers used were FNe1F: 5′TGGCTGTCAGTCAAAGCAAG3′ and FNi1R: 5′CAGCTGGTTTCTCTCAGTAAAGC3′. Results were expressed in relation to the pre-mRNA value of cells never treated with DRB.

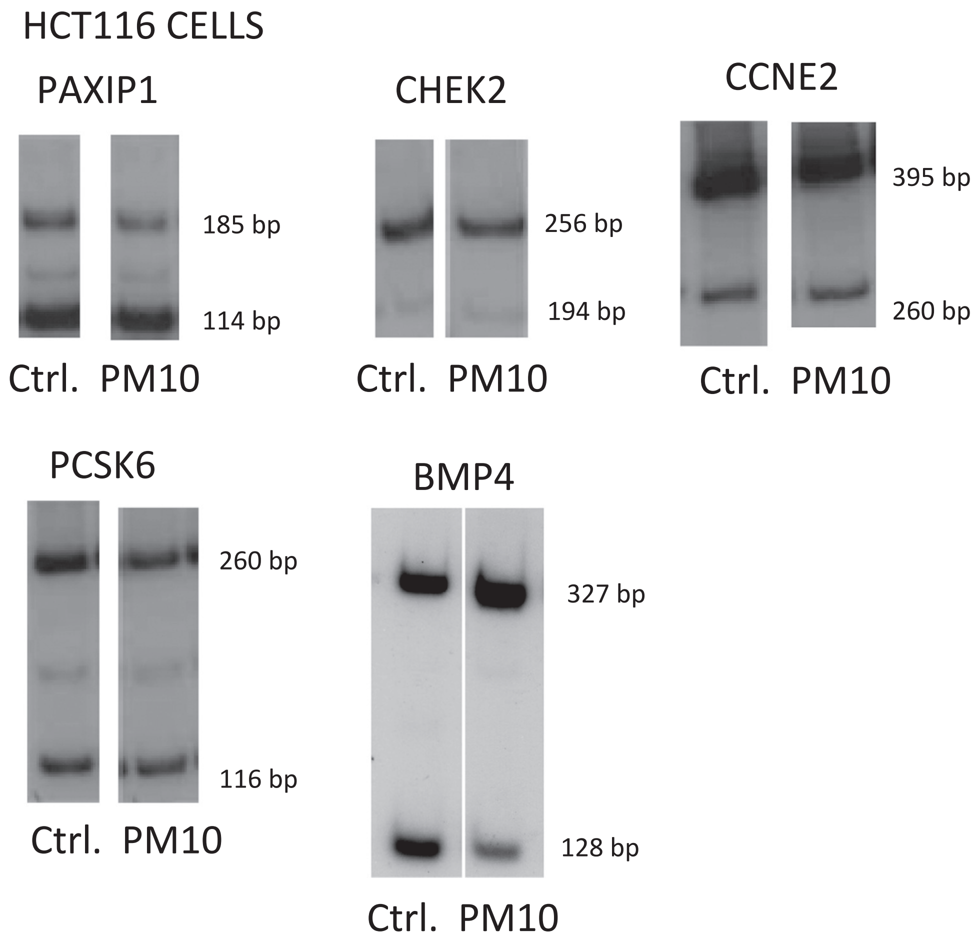

Fig. 2. Effects of PM10 particles on alternative splicing patterns in HCT116 cells of five cancer-related alternative splicing events (Klinck et al., 2008). RNA extraction and radioactive RT-PCR alternative splicing assays are described in Experimental Procedures. PAXIP1 (Pax interacting protein 1), Chek2 (CHK2 checkpoint homolog), CCNE2 (Cyclin E2), PCSK6 (Proprotein convertase subtilisin/kexin type 6) and BMP4 (Bone morphogenetic protein 4). HCT116 cells were incubated with 250 μg/ml of PM10 for 48 h.

3. Results

3.1. Cell viability

We first tested the effects of particulate dust (PM10) on cell viability using the human cell line HCT116 (human colon transformed), chosen as a model for colon cancer cells and the HEK293 line, from human embryo kidney, used as a control. Fig. 1 shows that increasing concentrations of dust cause cell death in both cell lines. However, HCT116 cell are much more resistant with a 50% lethal concentration of 400 μg/ml of dust against 60 μg/ml for the HEK293 cells. This result prompted us to investigate the putative effects of particulate dust on alternative splicing in HCT116 cells using dust concentrations lower that 400 μg/ml.

3.2. Alternative splicing

We investigated the effects of PM10 on five independent alternative splicing events (ASEs), corresponding to five different genes randomly chosen from a cancer-related-RT-PCR panel assessing 96 ASEs (Klinck et al., 2008). Fig. 2 shows that the ASEs from the human genes PAXIP1 (Pax interacting protein 1), Chek2 (CHK2 checkpoint homolog), CCNE2 (Cyclin E2) and PCSK6 (Proprotein convertase subtilisin/kexin type 6) are not affected by incubation of HCT116 cell with as much as 250 μg/ml of PM10 for 48 h. On the contrary, under similar conditions PM10 causes a conspicuous change in the alternative splicing pattern of the BMP4 (Bone morphogenetic protein 4) gene, with a 4–5-fold increase in the amounts of the longer mRNA isoform. The human BMP4 gene (Fig. 3A) has four exons. Two of its three introns interrupt the 5′ untranslated region (s), and the only ASE involves two alternative 5′ splice sites whose differential usage affects the length and sequence of the 5′UTR, but does not affect the sequence of the encoded protein. So, the two BMP4 mRNA splicing forms differ by the length of their 5′UTRs, being 419 or 210 nucleotides-long, and giving rise to the 327 and 128 bp-long RT-PCR products respectively. Therefore, treatment of cells with PM10 upregulates the long 5′UTR mRNA isoform but does not affect the sequence of the BMP4 protein.

3.3. Regulation of BMP4 alternative splicing

Alternative splicing is usually controlled by SR-proteins (Shepard and Hertel, 2009) that constitute a family of serine–arginine-rich splicing factors able to bind to specific sequences in pre-mRNAs, known as splicing enhancers and silencers. Fig. 3B shows that RNAi depletion of three of the most common SR proteins (SRSF1, 2 and 3, previously known as SF2/ASF, SC35 and SRp20), that individually or in combination affect the vast majority of ASEs, have no effect on BMP4 alternative splicing. This negative result led us to explore if BMP4 splicing was regulated instead by changes in transcriptional elongation, as previously reported for several ASEs (de la Mata et al., 2003; Schor et al., 2009, 2013; Alló et al., 2009; Ameyar-Zazoua et al. 2012; Dujardin et al., 2014) whose splicing is coupled to RNA polymerase II (Pol II) transcription.

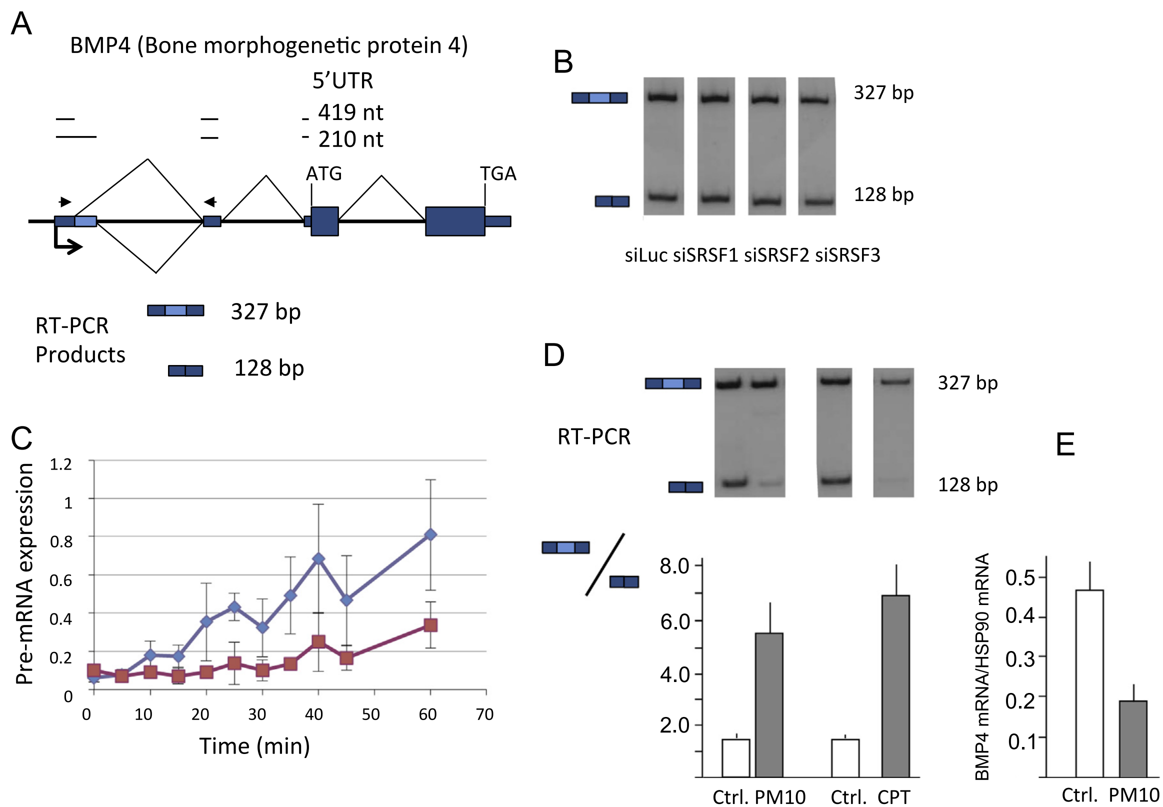

Fig. 3. Regulation of BMP4 alternative splicing. (A) Scheme of the human BMP4 gene and the alternative splicing event affecting its 5′ untranslated region (5′UTR). Arrows indicate the primers used for the alternative splicing radioactive RT-PCR assay. (B) Effects of RNAi depletion of three SR proteins in HCT116 cells on BMP4 alternative splicing. Transfections with siRNA's were performed as described in de la Mata et al. (2003). (C) Camptothecin (CPT) effectively inhibits RNA polymerase II elongation in HCT116 cells. Comparison of endogenous fibronectin pre-mRNA expression at an amplicon located approximately 500 bp downstream of the promoter in the presence (red line) or absence (blue line) of 6 μM CPT, after treatment with DRB, as explained in Experimental Procedures. (D) Comparison of the effects of PM10 and CPT on BMP4 alternative splicing. (E) Effect of PM10 on BMP4 mRNA levels with respect to Hsp90 mRNA levels used as control, determined using quantitative real time RT-PCR as described in Section 2. In all cases, HCT116 cells were incubated with 250 μg/ml of PM10 for 48 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

One of the ways to inhibit Pol II elongation is to treat cells with the DNA topoisomerase I inhibitor camptothecin (CPT). The mechanism through which CPT inhibits Pol II elongation is not completely known. However, it was recently shown that CPT is able to induce Pol II hyperphosphorylation (Dutertre et al., 2010; Dujardin et al., 2014) that is not accompanied by any qualitative or quantitative change in the abundance or phosphorylation of SR proteins. This indicates that the changes in alternative splicing caused by CPT are not due to an indirect effect of the drug on SR proteins. Because Pol II hyperphosphorylation after UV irradiation was shown to be the cause of changes in alternative splicing due to a reduction in Pol II elongation (Muñoz et al., 2009), it is very likely that CPT acts through a similar mechanism. Therefore, before assessing the effects of CPT on BMP4 alternative splicing, we first tested if CPT was able to inhibit Pol II elongation in HCT116 cells. For this, we compared Pol II elongation on an endogenous HCT116 model gene (FN1, encding fibronectin) in the absence and presence of CPT by reversibly blocking Pol II transcription with 300 mM 5,6-dichloro-1-beta-D-ribofuranosyl- benzimidazole (DRB) for 5 h and subsequent washing off of DRB. This DRB wash-off allows transcription to reinitiate, according to the procedure developed by Singh and Padgett (2009).

Therefore, before assessing the effects of CPT on BMP4 alternative splicing, we monitored the pre-mRNA levels through one RT-PCR amplicon, located approximately 500 bp downstream of the transcriptional promoter, at various time points after DRB wash. As shown in Fig. 3C, recovery of amplification was delayed for CPT-treated cells which clearly indicates that the drug effectively inhibits elongation in HCT116 cells. Similar CPT concentrations as those inhibiting Pol II elongation upregulate the long 5′UTR alternative splicing isoform of BMP4. In other words, both PM10 and CPT have similar qualitative and quantitative effects on BMP4 alternative splicing (Fig. 3D). These results suggest that the mechanism of action of PM10 on BMP4 alternative splicing involves an inhibition of Pol II elongation. To partially support this possibility we tested whether PM10 was able to specifically inhibit BMP4 mRNA levels, a consequence of a putative inhibition of BMP4 transcription. This prediction was confirmed by showing that PM10 reduces total BMP4 mRNA levels either measured by quantitative real time RT-PCR with primers mapping to a region common to both splicing isoforms (Fig. 3E) or through semi-quantitative end-point RT-PCR with increasing number of cycles (Fig. 4A and B). Both methods revealed approximately a 2.3-fold reduction in total BMP-4 mRNA levels. Furthermore, we found that the reduction in total BMP-4 mRNA levels causes a concomitant reduction in BMP-4 protein levels measured by Western blotting (Fig. 4C).

4. Discussion

We show here that incubation of a colon cancer human cell line with particulate air pollutants including particles of 10 μm diameter or less (PM10) causes an important change in alternative splicing (Figs. 2 and 3D) of the mRNAs encoding the growth factor BMP4, by favoring a splicing isoform that does not alter the encoded amino acid sequence but that reduces the overall expression of the protein (Figs. 3E and 4).

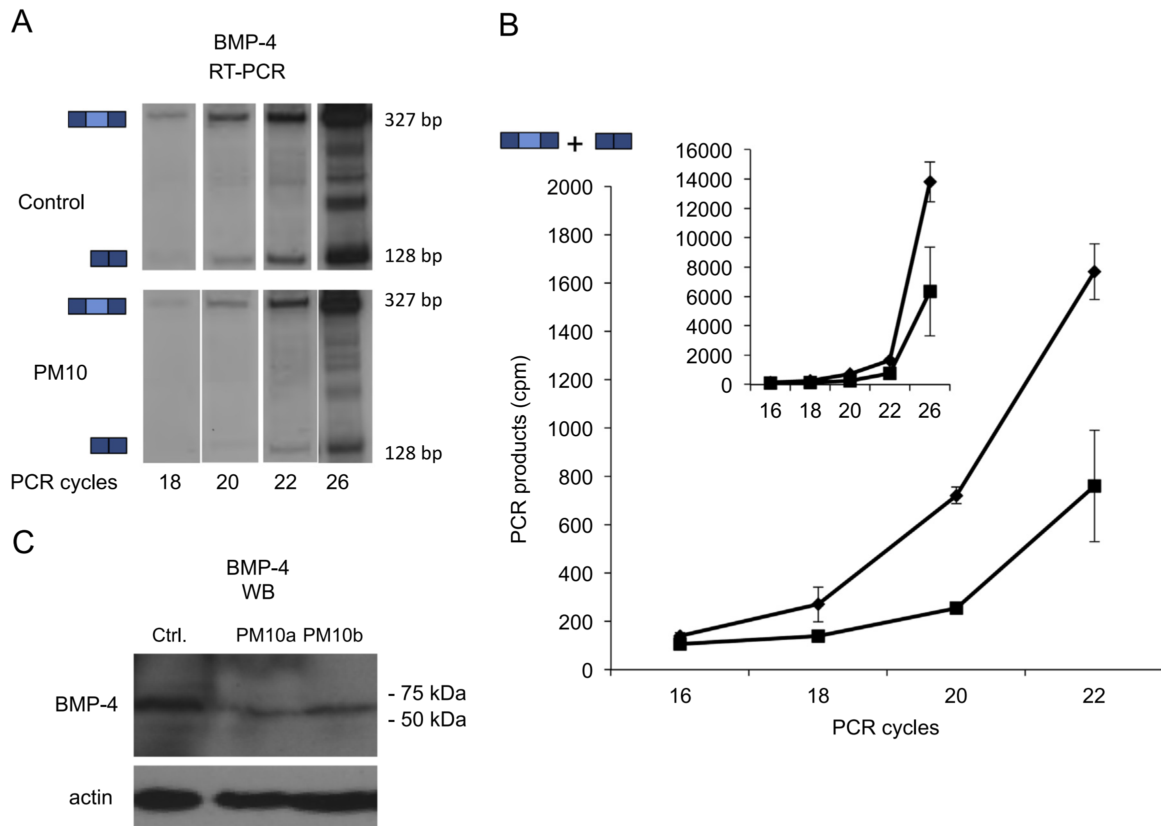

Fig. 4. PM10 particles inhibit BMP-4 total mRNA (A, B) and protein (C) expression in HCT116 cells. (A) RNA extraction and radioactive RT-PCR alternative splicing assays are described in Experimental Procedures, with the only difference that cDNA samples were obtained at different numbers of PCR cycles. (B) Quantifications of radioactive gels in A. (C) Western blot of total protein extracts from HCT116 revealed with antibodies to BMP-4 (N-16, sc-6896) and to actin as loading control (C-4, sc-47778), both from Santa Cruz Biotechnologies. PM10a and PM10b denote separated tretment of cells with two different PM10 extraction samples.

An important issue to discuss is how PM10 enters the cells in culture. Particle uptake by cells has been reported to occur by phagocytosis of insoluble parts and/or pinocytosis of soluble parts (Saxena et al., 2008; Nemmar et al., 2013).

BMP4 is a member of the bone morphogenetic protein family which is part of the transforming growth factor-beta superfamily that includes large families of growth and differentiation factors. Bone morphogenetic proteins were originally identified by an ability of demineralized bone extract to induce endochondral osteogenesis in vivo in an extraskeletal site. This particular family member plays an important role in the onset of endochondral bone formation in humans, and a reduction in expression has been associated with a variety of bone diseases, including the heritable disorder Fibrodysplasia Ossificans Progressiva.

Our results are consistent with a role of BMP4 in preventing colon cancer development. First of all, BMP4 gene variants have been shown to predispose to colorectal cancer (Kallioniemi, 2012). Besides, whereas BMP4 expression seems to be absent in colon cancer stem cells, its overexpression was shown to promote terminal differentiation, apoptosis, and chemosensitization of these cells present in tumors, which led to the proposal that BMP4 might be developed as a therapeutic agent against cancer stem cells in advanced colorectal tumors (Lombardo et al., 2011). This suggests that a reduction in BMP4 expression caused by the ingestion of air pollutants present in the PM10 fraction might contribute to the onset of colon cancer. We think that the downregulation of BMP4 expression caused by PM10 (Figs. 3E and 4) is the consequence of the actual change in alternative splicing reported here.

Indeed, genome-wide studies have revealed that approximately 35% of human genes contain introns within the 5′UTR whose presence enhances their expression (Cenik et al., 2010). The presence of 5′UTR introns allows for alternative splicing. Tissue-dependent alternative splicing within 5′UTRs is common and can be functionally important, most probably by reducing translation efficiency through differential inclusion of upstream AUGs (uAUGs), as demonstrated for the human NOD2 gene (Rosenstiel et al., 2007). In agreement with these observations, inclusion of the 209-nt differential segment in the longer BMP4 5′UTR mRNA isoform introduces an uAUG within the sequence ccccgAUGG that fits (underlined bases) the Kozak consensus for functional translation initiation codons (Kozak, 1986).

In summary, we believe that PM10 downregulates BMP4 expression in colon cancer cells through the reduction in mature mRNA levels and translational inhibition as a consequence of the upregulation of the long 5′UTR mRNA bearing a functional uAUG. To what extent this inhibition effectively contributes to colorectal pathologies including cancer will deserve further investigation.

Our results also provide insights on the mechanism by which PM10 regulates BMP4 alternative splicing by showing that similar effects can be caused by agents that inhibit transcriptional elongation in HCT116 cells. Several studies have demonstrated that changes in elongation can affect alternative splicing decisions of specific ASEs framed in the so-called kinetic coupling model (Kornblihtt, 2007, 2013).

The fact that only a subset of ASEs is affected by elongation (Muñoz et al., 2009; Ip et al., 2011) might explain why PM10 affects the BMP4 gene alternative splicing, but not the splicing of the other five genes in the panel (Fig. 2). It is worth-noting that, external stress-inducing cues producing DNA damage, including UV-irradiation, have been shown to regulate ASE through the kinetic coupling (Muñoz et al., 2009; Ip et al., 2011), which calls for

further investigation to see if the ambient PM10 effect on alternative splicing is related to its reported genotoxicity (El-Assouli et al., 2007). If, like UV-irradiation, PM10 were causing DNA damage, the mechanism for the effect reported here would involve Pol II hyperphosphorylation (Muñoz et al., 2009), something that remains to be explored.

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