

Downregulation of microRNA-126 augments DNA damage response in cigarette smokers and COPD patients

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To the editor:

Increased DNA damage response (DDR) contributes to the pathophysiology of ageing disorders including chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD) and cancer (1, 2). DNA damage due to cigarette smoke-induced oxidative-stress, activates the DDR signaling pathway, controlled mainly by the ataxia-telangiectasia-mutated (ATM) protein-kinase. Accumulation of DNA damage promotes cellular senescence and disrupts tissue homeostasis (2). Using circulating endothelial precursors called blood outgrowth endothelial cells (BOEC) or endothelial-colony-forming-cells, we have previously demonstrated increased DDR, endothelial senescence and dysfunction in smokers and COPD patients (3), supporting the concept of accelerated ageing of the endothelium as contributing to CVD in these groups.

MicroRNAs are important regulators of almost every cellular process including DNA repair. MiR-126 (miR-126-3p), a microRNA enriched in endothelial cells, plays a key role in angiogenesis and vascular homeostasis (4). MiR-126 is reduced in patients susceptible to develop or with CVD (5-7), and in several types of cancer, including lung cancer (8). The link between miR-126 and DDR signaling in COPD is currently unknown. Therefore, we investigated whether miR-126 is reduced by cigarette-smoke *in vivo* and in endothelial and lung epithelial cells from COPD patients, and its link to DDR activation.

Methods

To investigate DDR due to cigarette-smoke *in vivo*, male C57BL/6 mice were challenged for 28 days with cigarette-smoke or ambient-air as previously described (9). BOEC were isolated from human blood samples, and human primary bronchial epithelial cells were

isolated from lung tissue as described (3, 10). Informed consent was obtained from all individuals (Table 1). Data have been derived from subjects and animals used in previous reports (3, 9, 10). Human umbilical vein endothelial cells (HUVEC) were transfected with miRCURY locked-nucleic-acid microRNA inhibitor hsa-miR-126-3p (Exicon, Vedbaek, Denmark, #4109148-001) or negative control (#199006-001) at 50nM. RNA was extracted using the mirVana Isolation Kit (Ambion) or the RNeasy kit (Qiagen). MiR-126-3p levels, and RNU44 and snoRNA135 as reference genes, were measured by TaqMan assays (#477887, #4427975, #4427975, Life Technologies) (7). Levels of ATM, SPRED-1 and p85 normalized to GAPDH mRNA were measured by quantitative real-time polymerase-chain-reaction (RT-PCR). Primer sequences are available on request. Immunofluorescence was performed with the phospho-(Ser/Thr) ATM/ATR substrate antibody (Cell Signaling Technology, New England Biolabs, Hertfordshire, UK). Nuclei were visualized using Draq-5 (Biostatus Limited, Leicester, UK). The following antibodies were used for Western blotting: ATM (Cell Signaling Technology), phospho-ATM-(Ser1981) (Millipore), α -tubulin (Sigma-Aldrich Company Ltd). Statistical analysis was performed with GraphPad Prism 7. Data are expressed as mean \pm SEM. Comparisons between groups were performed with one-way ANOVA, Student's t-tests or Mann-Whitney U test according to normality of the data. Correlations were estimated with the Spearman r-correlation coefficient.

Results

The ATM protein-kinase is rapidly phosphorylated and activated in response to DNA double-strand breaks and regulates the DDR (2). ATM-phosphorylation was significantly increased in BOEC from COPD patients compared to non-smokers (Figure 1A), suggesting activation of ATM signaling pathway in COPD. We next investigated whether ATM

activation is linked to miR-126 dysregulation. MiR-126 levels were reduced in BOEC from smokers and COPD patients compared to non-smokers (Figure 1B), suggesting that cigarette-smoke downregulates miR-126 expression in the endothelium and could contribute to the aberrant activation of DDR in these groups.

Subsequently, we examined whether miR-126 regulates ATM protein-kinase and DDR. As previously shown (4), inhibition of miR-126 function in HUVEC increased the expression of p85 and SPRED1 (Figure 1C). Under the same conditions, ATM mRNA and protein levels were increased (Figure 1C), suggesting that miR-126 controls DDR by repressing ATM expression.

We then used a mouse model of sub-chronic exposure to cigarette-smoke, to confirm our findings *in vivo*. We found that miR-126 levels were reduced in lungs from mice exposed to cigarette-smoke (Figure 1D). Within the same samples, there was also increased ATM expression and activity (Figure 1E-F), in line with our *ex-vivo* findings in patients' endothelial cells.

As miR-126 was dysregulated in whole lung tissue in our *in vivo* model, we investigated whether miR-126 expression is affected in lung epithelial cells in COPD. Interestingly, we found reduced miR-126 levels in lung epithelial cells from COPD patients (all current or ex-smokers) compared to non-smokers (Figure 1G). Additionally, miR-126 levels in lung epithelial cells negatively correlated with smoking history assessed as pack-years (Spearman r : -0.73, $p < 0.01$) and positively correlated with disease severity measured as FEV₁% predicted (Spearman r : 0.57, $p < 0.05$), suggesting that miR-126 expression is down-regulated with extended exposure to cigarette-smoke and increased severity of lung disease.

These results identify a novel miR-126-dependent pathway controlling DDR caused by cigarette-smoke, where down-regulation of miR-126 enhances ATM activation and thereby promotes tissue ageing and dysfunction.

Discussion

In this study, we demonstrate that chronic exposure to cigarette-smoke causes reduced expression of miR-126 and increases the DDR. Using a combined *in vivo* and *ex-vivo* approach, namely mice exposed to cigarette-smoke and endothelial and lung epithelial cells from COPD patients, we show that exposure to cigarette-smoke down-regulates miR-126 expression.

MiR-126 is critical for endothelial function, and miR-126 supplementation has been proposed therapeutically in *in vivo* models of vascular injury and pulmonary hypertension (5, 6). We have previously shown that BOEC from both smokers and COPD patients exhibit increased DDR, senescence and dysfunction (3); these molecular defects could contribute to CVD, a major cause of mortality in these individuals. Here we demonstrate that miR-126 is down-regulated in BOEC and in lung epithelial cells from COPD patients, highlighting miR-126 as a possible contributor to endothelial and lung tissue dysfunction and thus potential therapeutic target for COPD.

We also show that miR-126 controls the DDR by repressing the ATM protein-kinase activity in endothelial cells. This is important as it highlights a novel pathogenetic pathway of miR-126 in age-related diseases, including cancer. Further studies will show whether restoration of miR-126 has a beneficial effect on lung and vascular function in COPD, by regulating the aberrant activation of ATM and downstream molecular pathways that promote accelerated lung and vascular ageing.

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Figure Legends

Figure 1. miR-126 is downregulated and enhances ATM activation in smokers and COPD patients and in an *in vivo* model of sub-chronic cigarette-smoke exposure.

(A) Activation of ATM was assessed by measuring phosphorylation of ATM/ATR substrate by immunofluorescence staining (n=3). Draq5 was used for nuclear staining. The number of nuclear foci per nucleus was quantified with Volocity software in at least 5 optic fields (z-stack images). An increased number of cells with focal nuclear staining of p-ATM/ATR was observed in COPD patients compared to non-smokers. Arrows show nuclei with increased number of nuclear foci (scale bars 20 μ m). **(B)** We measured miR-126 by TaqMan RT-PCR in BOEC from non-smokers (n=7), healthy smokers (n=6) and COPD patients (n=5). RNU44 was used for normalisation. **(C)** HUVEC were treated with specific anti-miR-126 inhibitor vs control for 72h (baseline conditions, no growth factors). mRNA levels for SPRED1 and p85 (positive controls for miR-126 inhibition), and for ATM were measured by RT-PCR. ATM protein levels were quantified by western blotting. GAPDH and α -tubulin were measured for normalization. **(D-F)** Male C57BL/6 mice were challenged for 28 days with cigarette-smoke (for 1 hour twice daily, 4 hours apart) or to ambient air. **(D)** MiR-126 was measured by TaqMan RT-PCR in lung tissue from mice exposed to cigarette-smoke (n=6) vs control mice lung tissue (n=5). Sno135 was measured for normalization. **(E-F)** ATM mRNA levels were measured by RT-PCR and activation of ATM (phospho-ATM) and ATM protein levels were measured by western blotting in lung tissue from mice exposed to cigarette-smoke (n=6) vs control mice (n=6). GAPDH and α -tubulin were measured for normalization. **(G)** MiR-126 was measured by TaqMan RT-PCR in lung epithelial cells from non-smokers (n=6) and COPD patients (n=6). RNU44 was used for normalization. *p<0.05, **p<0.01

Table 1: Clinical characteristics of volunteers

Blood outgrowth endothelial cells (BOEC)	Healthy non smokers	Healthy smokers	COPD
Number (n)	7	6	5; (4 moderate; 1 severe)*
Sex (M/F)	4/3	4/2	5/0
Age (years)	54±4	57±4	69±3 [†]
Smoking (pack-years)	0	37±10	68±38(3 ex-smokers)
FEV₁, % predicted	99.1±6.4	93.6±3.8	61.4±5.8 ^{‡,§}
FEV₁/FVC, %predicted	77.9±0.8	71.6±3.6	53±5.2 ^{,§}

Lung epithelial cells	Healthy non smokers	COPD
Number (n)	6	6; (3 moderate; 1 severe; 2 very severe)*
Sex (M/F)	0/6	5/1
Age (years)	63±6	66±2
Smoking (pack-years)	0	47±5(3 ex-smokers)
FEV₁, % predicted	97.2±5.6	47.2±10.5
FEV₁/FVC, %predicted	75.2±3.4	42.02±9.2

Values are expressed as means ± SEM. COPD indicates chronic obstructive pulmonary disease; *staging of COPD is according to the Global initiative for chronic Obstructive Lung Disease (GOLD) criteria; M = male; F = female; pack-years = number of packs cigarettes smoked per day multiplied by the number of years of smoking; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; FEV₁ and FEV₁/FVC ratio are post bronchodilator for subjects with COPD, smokers or non-smokers; [†] p<0.05 (comparison between non-smokers and COPD); [‡] p<0.001 (comparison between non-smokers and COPD); [§] p<0.01 (comparison between smokers and COPD); ^{||} p<0.01 (comparison between non-smokers and COPD).

Figure 1

