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TNFα induces the expression of genes associated with endothelial dysfunction through p38MAPK-mediated down-regulation of miR-149

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ABSTRACT

MicroRNAs have been proposed as novel regulators of vascular inflammation and dysfunction. This study aimed to evaluate the role of miR-149 in regulating the expression of key molecules associated with TNFα-induced endothelial activation. miR-149 was selected by *in silico* analysis and microRNA target prediction. Endothelial dysfunction was induced by TNFα treatment in Eahy926 endothelial cells and HUVEC. miR-149 level was evaluated by quantitative real time-polymerase chain reaction (RT-qPCR). Metalloproteinase-9 (MMP-9) was measured by zymography, Inducible Nitric Oxide Synthase (iNOS) by immunoblotting, Interleukin-6 (IL-6) and Interleukin-8 (IL-8) by ELISA. miR-149 regulatory effect was evaluated by gain-of-function technique upon miR-149 mimics transfection.

TNFα down-modulated miR-149 level in Eahy926 and HUVEC. This effect was significantly abolished in Eahy926 by treatment with p38MAPK inhibitor. miR-149 mimic transfection counteracted the TNFα-induced expression of MMP-9, iNOS and IL-6. No effect was detected on IL-8 expression. Our results suggest that miR-149 represents an important new regulator of endothelial function through negative regulation of molecules associated with TNFα-induced endothelial dysfunction.

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1. Introduction

TNFα plays a pivotal role in the initiation and progression of vascular disorders by modulating the expression of molecules involved in vascular tone, inflammation and remodelling, thus inducing endothelial dysfunction [1–3]. The molecular mechanism underlying TNFα effects on endothelial function is well described but the role of microRNAs (miRNAs) is not fully elucidated. miRNAs are a class of non-coding small RNAs that negatively regulate the expression of proteins at the post-transcriptional level. The dysregulated expression of specific miRNAs is associated with different cardiovascular diseases and with pathological conditions mediated by pro-inflammatory stimuli, including TNFα-induced endothelial injury [4–7]. However, the complete patterns of miRNAs regulated by TNFα and involved in endothelial dysfunction remain to be fully elucidated. Bioinformatics analysis predicted that miR-149 would target several important genes related to endothelial dysfunction, including Metalloproteinase-9 (MMP-9), Inducible Nitric Oxide Synthase (iNOS), Interleukin 6 (IL-6) and Interleukin 8 (IL-8). These evidences supported us to focus on miR-149 in an inflammatory cellular model of endothelial dysfunction induced by TNFα. We investigated the expression profile of miR-149 in human endothelial cells subjected to TNFα and the signaling pathway involved in miR-149 regulation. We also evaluated miR-149 regulatory potential on markers involved in endothelial dysfunction such as MMP-9, iNOS, IL-6 and IL-8.

2. Materials and methods

2.1. MicroRNA target prediction

miRNA binding sites and the mirSVR score were predicted using miRanda software (http://www.microrna.org/microrna/home.do). mirSVR is an algorithm for scoring and ranking the efficiency of miRanda-predicted microRNA target sites. The scores can be interpreted as an empirical probability of down-regulation of a single gene by a specific microRNA [8]. "Good" mirSVR score refers to

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miRNA targets with <−0.1 score, and "non-good" mirSVR score refers to targets with >−0.1 score.

2.2. Cell culture condition and treatments

Primary human umbilical vein endothelial cells (HUVEC) were cultured in gelatin-coated flasks using M199, 10% fetal calf serum supplemented with FGF (1 μg aFGF ± 1 μg bFGF/100 ml medium), EGF (1 μg/100 ml medium), heparin (10 mg/100 ml medium) and hydrocortisone (0.1 mg/100 ml medium). Eahy926 endothelial cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. For treatment experiments, cells were set in serum-free medium for 2 h, and then treated with TNFα (100 ng/ml, Sigma–Aldrich, USA, T6674) for 2 or 24 h, as indicated. In the experiments with the p38MAPK inhibitor SB203580 (20 μM, Santa Cruz, USA, sc-3533), JNK inhibitor SP600125 (20 μM, BioMol, USA, BML-EI305R), and Akt inhibitor (40 μM, Calbiochem, USA, 124005), cells were pretreated with inhibitors for 1 h. Cells were lysed in miRNA lysis buffer (Ambion, Inc., Austin, TX, USA) for miRNA extraction and conditioned media (CMs) were collected.

2.3. MicroRNA preparation and quantitavive reverse transcription PCR (RT-qPCR)

miRNAs were isolated from cultured cells using the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. After RNA quality control, RT-qPCR was performed using a TaqMan miRNA Reverse Transcription Kit and a miR-149 custom design TaqMan Small RNA Assay (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The qPCR reaction was performed in triplicate with 10 ng RNA, using U6 RNA as housekeeping. Data were analysed with the ΔΔct method, relatively to a not-treated control.

2.4. miR-149 mimics transfection

Eahy926 endothelial cells were transfected using Lipofectamine RNAiMax Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Briefly, cells were transfected with mirVana™ miR-149 mimic (M-miR149) (Ambion) or with mirVana™ miRNA mimic Negative Control #1 (C-miRNA) (Ambion) as validated negative control at three different concentration (30, 60 and 100 nM). Successful transfection (>85% of all cells) was confirmed by visual fluorescent analysis after transfection with Block-iT™Alexa Fluor fluorescent oligo. After 48 h, cells were treated with TNFα (100 ng/ml) for 6 and 24 h and then lysed in RIPA buffer (5 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS). CMs were collected for zymography and ELISA.

2.5. Zymography for MMP-9

Equivalent protein amounts (by BCA methods) from CM were run on SDS-acrylamide gel cast with 0.28% w/v gelatin (type A), rinsed twice for 30 min in 2.5% Triton X-100, incubated for 16–18 h at 37 °C in 40 mm Tris–HCl, 0.2 m NaCl, 10 mm $CaCl_2$, stained with 0.2% Coomassie blue and destained in 50% methanol, 10% acetic acid.

2.6. Western blotting

Fifty micrograms of proteins from lysates were run on reducing SDS-acrylamide gels and electrotransferred to nitrocellulose. Membranes were saturated at room temperature for 1 h in TTBS (20 mm Tris–HCl pH 7.5, 500 mm NaCl, 0.01% Tween 20, 5% non-fat milk). The membranes were incubated with primary antibody for 16 h, then with horseradish peroxidase-secondary antibody (GE Healthcare Europe GmbH) for 1 h. The bands were visualized by ECL chemiluminescence (Merck Millipore, Darmstadt, Germany). The membranes were stripped and re-blotted with polyclonal actin. Bands were quantified by optical densitometry (gel analysis system GeneGenius, Syngene, Cambridge, UK). The primary antibodies used were: polyclonal anti-iNOS (Santa Cruz, USA, sc-651 dilution 1:200), polyclonal anti-actin (Santa Cruz, sc-1615, 1:1,000 dilution).

2.7. IL-6 and IL-8 levels

IL-6 and IL-8 level was measured in CM by Instant ELISA immunoassay according to manufacturer instructions (BMS213INST and BMS204/3INST, Bender Med System, Vienna, Austria).

2.8. Statistical evaluation

Each experiment was repeated three to five times. Data shown are mean ± SE. The statistical significance of the results was determined using ANOVA followed by Fisher test. $P < 0.05$ was considered significant.

3. Results and discussion

Many risk factors, such as aging, smoking, inflammation, hyperlipidemia and hyperglycemia, may contribute to the pathogenesis of vascular disease through modulation of TNFα signaling. It is well known that TNFα plays a pivotal role in the start and progression of vascular diseases by inducing endothelial dysfunction. In order to better understand the molecular mechanism underlying endothelial dysfunction, we chose to treat human endothelial cells with TNFα (100 ng/ml) and to investigate the role of miR-149 in vascular activation. The induction of endothelial dysfunction upon TNFα treatment was monitored in Eahy926 and HUVEC by measuring the expression of specific secreted markers including MMP-9 and IL-6 (Fig. 1A and B). The level of miR-149 was evaluated in the same cultures after 2 or 24 h by RT-qPCR. miR-149 was expressed by both endothelial cell lines and its level was decreased by TNFα treatment. In Eahy926 cells, miR-149 down-regulation was dramatic after 24 h, up to 70% of control (Fig. 1C). Moreover, in HUVEC the level of endogenous miR-149 was higher than in Eahy926 cell line (data not shown) and was down-regulated by TNFα, up to 40% of control (Fig. 1D). In a previous work we showed that TNFα exerts its action on Eahy926 cells through the modulation of different signaling pathways, including p38MAPK, JNK and Akt [3]. In order to evaluate which of these pathways is involved in miR-149 deregulation by TNFα, we performed experiments with chemical inhibitors of p38MAPK (SB203580), JNK (SP600125) and Akt. Treatment with SB203580 significantly counteracted the TNFα-induced decrease of miR-149. No effects were detected in presence of JNK and Akt inhibitors (Fig. 1E). These data suggest that TNFα down-regulates miR-149 through p38MAPK signaling activation.

To assess the role of miR-149 in endothelial dysfunction, a computational analysis was performed in order to identify putative target genes for miR-149. The *in silico* analysis has predicted miR-149 target sequences in 3′-UTR of MMP-9, iNOS, IL-6 and IL-8 (Fig. 2). Therefore, miR-149 regulatory effect on MMP-9, iNOS, IL-6 and IL-8 was evaluated by gain-of-function technique upon miR-149 mimics (M-miR149) transfection. Eahy926 endothelial cells were transfected with three different concentrations of M-miR-149 (30, 60 and 100 nM) or miRNA mimic Negative Control (C-miRNA) for 48 h and then stimulated with TNFα for 6 or 24 h. Cell viability was measured in each experimental conditions by Trypan Blue exclusion method and no detrimental effect of transfection was

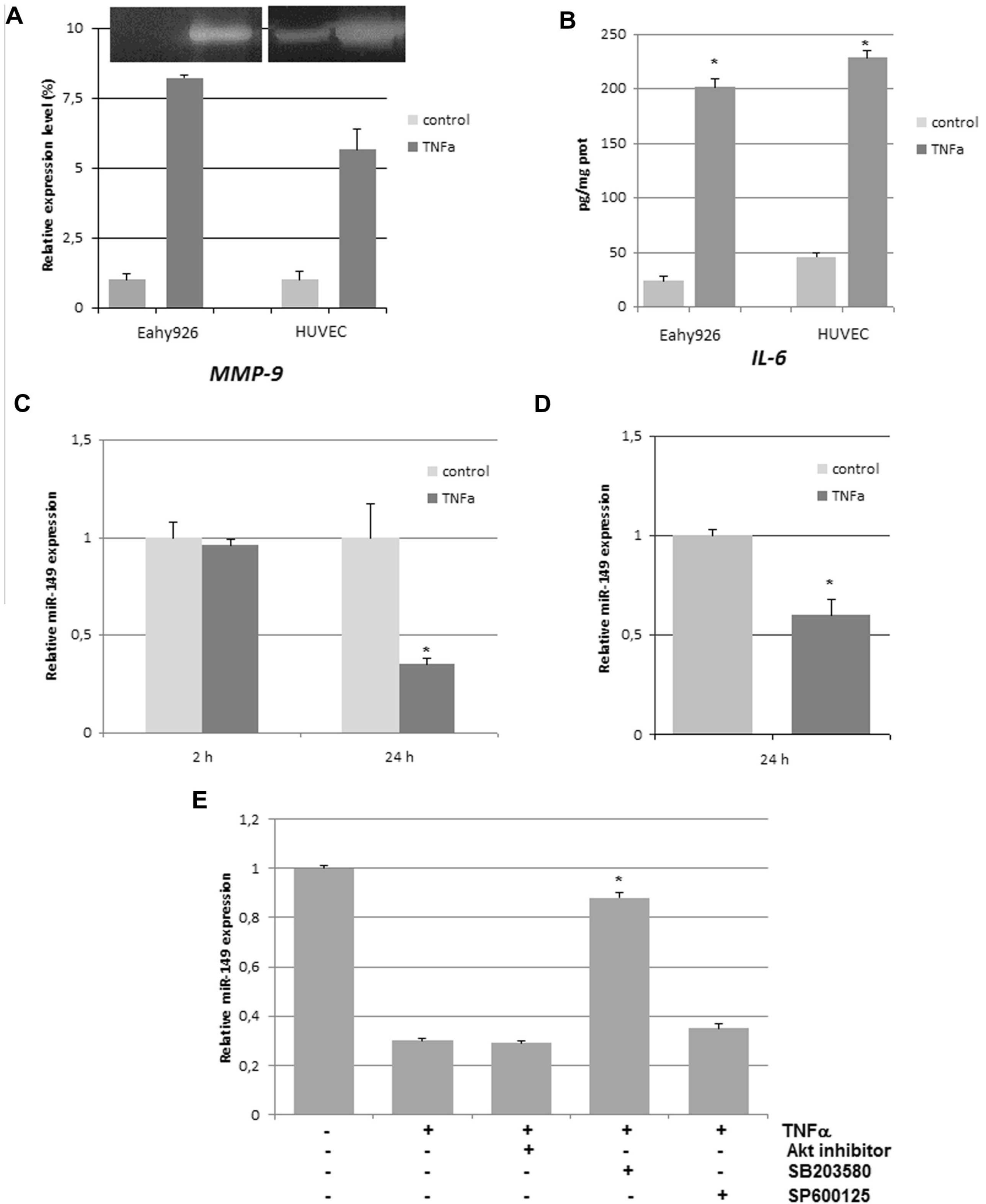

Fig. 1. TNFα induces endothelial dysfunction and down-regulates miR-149 level. Eahy926 cell line and HUVEC were treated with TNFα (100 ng/ml) for 2 or 24 h. Endothelial dysfunction was monitored by evaluating the expression of MMP-9 (A) and IL-6 (B) after 24 h. miR-149 level was evaluated in Eahy926 by RT-qPCR after 2 and 24 h (C) and in HUVEC after 24 h (D). miR-149 level was then evaluated in Eahy926 after treatment with p38MAPK inhibitor (SB203580), JNK inhibitor (SP600125) and Akt inhibitor (E). $^{*}p$ Value $\leqslant 0.05$.

detected (data not shown). As expected, TNFα induced MMP-9 expression (Fig. 3A and B). Transfection with M-miR149 lowered in a dose dependent manner MMP-9 expression while C-miRNA was ineffective. Similar results were obtained for iNOS and IL-6 expression, both induced by TNFα treatment. M-miR149 at the concentration of 100 nM restored to basal level the TNFα-induced

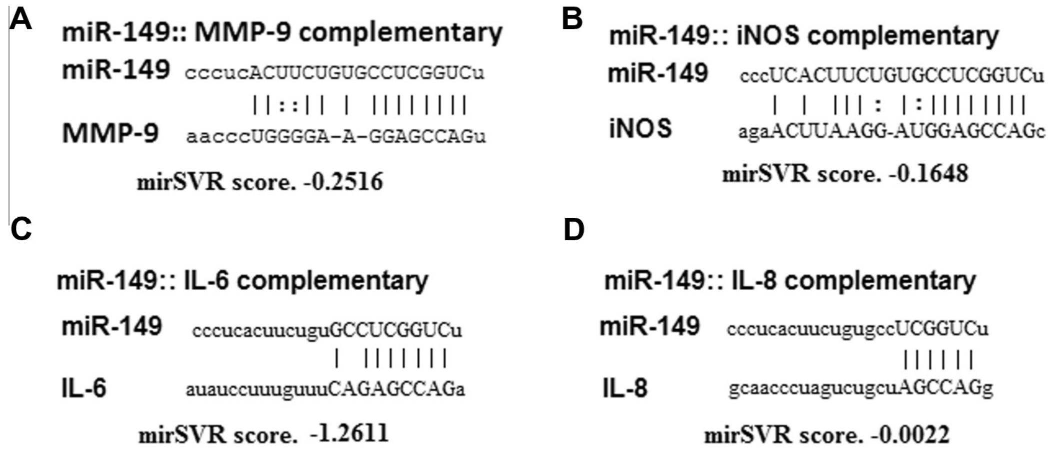

Fig. 2. miR-149 target prediction. The position of miR-149 target sites along 3′UTR of MMP-9 (A), iNOS (B), IL-6 (C) and IL-8 (D) was predicted by miRanda software. The mirSVR score is reported as the empirical probability of down-regulation of a specific gene by miR-149. "Good" mirSVR score refers to miRNA targets with <−0.1 score, and "non-good" mirSVR score refers to targets with >−0.1 score.

Fig. 3. miR-149 negatively regulates the TNFα-induced expression of MMP-9 and iNOS. Eahy926 cells were treated with mirVana™ miR-149 mimic (M-miR149) or with mirVana™ miRNA mimic Negative Control #1 (C-miRNA) at three different concentration (30, 60 and 100 nM). After 48 h, cells were treated with TNFα (100 ng/ml) for 6 or 24 h, as indicated. Endothelial cell production of MMP-9 was measured by gel zymography (A and B) and iNOS expression by immunoblotting (C and D). *p Value $\leqslant$0.05.

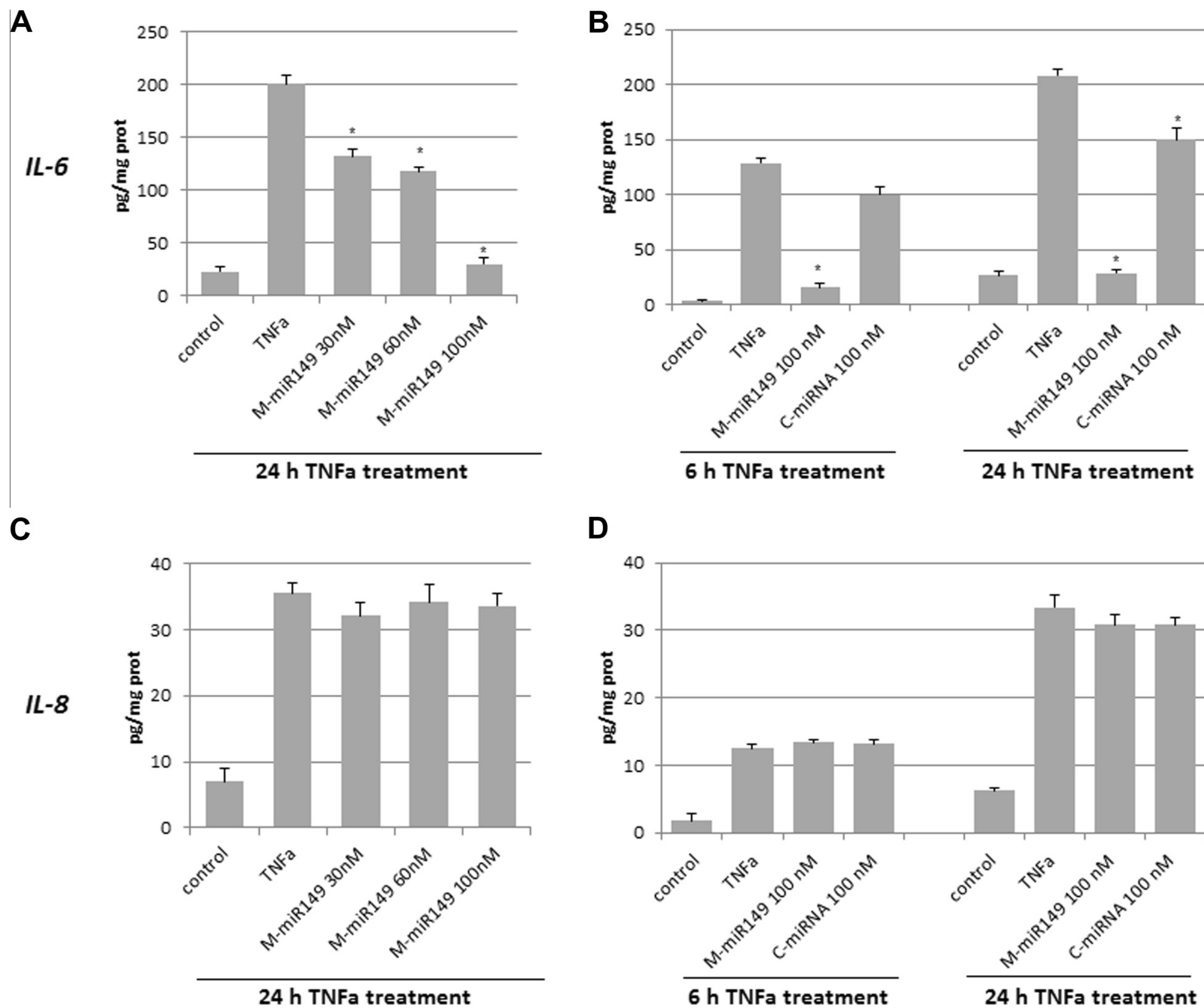

Fig. 4. miR-149 negatively regulates the TNFα-induced expression of IL-6, nor of IL-8. Eahy926 cells were treated with mirVana™ miR-149 mimic (M-miR149) or with mirVana™ miRNA mimic Negative Control #1 (C-miRNA) at three different concentration (30, 60 and 100 nM). After 48 h, cells were treated with TNFα (100 ng/ml) for 6 or 24 h, as indicated. Endothelial cell production of IL-6 (A and B) and IL-8 (C and D) was measured by ELISA. $^{*}p$ Value $\leqslant 0.05$.

expression of iNOS (Fig. 3C and D). Moreover M-miR149 transfection led to a significant dose-dependent decrease in IL-6 expression (Fig 4A and B). Conversely, no effect was detected on IL-8 expression (Fig. 4C and D).

On the basis of our findings, we can conclude that miR-149 represents an important new regulator of endothelial function. We show for the first time that miR-149 is constitutively expressed by endothelial cells and that is sensitive to TNFα treatment. Deregulation of miR-149 has been reported to be involved in several pathologies [9–12] but to our knowledge, this is the first evidence of miR-149 down-regulation in endothelial cells. TNFα down-regulated the level of miR-149 in both Eahy926 and HUVEC cell lines, suggesting that miR-149 down-regulation reflects a specific response of endothelial cells to TNFα. It should be noted that the modulation of miR-149 by TNFα may not be restricted to endothelial cell. A recent work reported miR-149 down-regulation in an inflammatory model of osteoarthritis induced by TNFα in human primary chondrocytes and chondrosarcoma cells [13]. Thus, it is likely that miR-149 down-modulation is a general downstream effect of TNFα treatment.

We also provide evidence that miR-149 negatively regulates the expression of three putative target genes, MMP-9, iNOS and IL-6, suggesting that some of the aspects related to the TNFα-induced endothelial activation might be attributable to miR-149 down-regulation. Conversely, miR-149 does not seem to have any effect on IL-8 expression. This result may be due to the experimental condition used (timing and M-miR149 concentration used in the transfection experiments). However the mirSVR score predicted for IL-8 was a "non good" mirSVR score suggesting a lower probability of IL-8 regulation by miR-149.

Our previous results suggested that TNFα induces endothelial dysfunction through the activation of many different non-overlapping pathways [2,3]. We here show that p38MAPK pathway mediates the effect of TNFα on miR-149 level, that in turn is a negative regulator of MMP-9, consistently with our previous study in which it was showed the involvement of p38MAPK in MMP-9 modulation.

In conclusion, the present study suggests that miR-149 protects against endothelial dysfunction through negative regulation of its functional target genes, MMP-9, iNOS and IL-6. These findings may have relevant implications for the treatment and the diagnosis of vascular disorders related to TNFα. miRNA delivery designed to mimic the function of endogenous miRNAs could represent a new tool for therapeutic intervention, useful to counteract endothelial

activation by TNFα. Furthermore, an intriguing perspective is to verify the possible use of miR-149 as clinical predictive serum biomarker for endothelial dysfunction and vascular diseases.

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