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# Deregulation of hsa-miR-20b expression in TNF-α-induced premature senescence of human pulmonary microvascular endothelial cells

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ABSTRACT

miRNAs are important regulators of cellular senescence yet the extent of their involvement remains to be investigated. We sought to identify miRNAs that are involved in cytokine-induced premature senescence (CIPS) in endothelial cells. CIPS was established in young human pulmonary microvascular endothelial cells (HMVEC-Ls) following treatment with a sublethal dose (20 ng/ml) of tumor necrosis factor alpha (TNF-α) for 15 days. In parallel, HMVEC-Ls were grown and routinely passaged until the onset of replicative senescence (RS). Differential expression analysis following miRNA microarray profiling revealed an overlapped of eight deregulated miRNAs in both the miRNA profiles of RS and TNF-α-induced premature senescence cells. Amongst the deregulated miRNAs were members of the miR 17–92 cluster which are known regulators of angiogenesis. The role of hsa-miR-20b in TNF-α-induced premature senescence, a paralog member of the miR 17–92 cluster, was further investigated. Biotin-labeled hsa-miR-20b captured the enriched transcripts of retinoblastoma-like 1 (RBL1), indicating that RBL1 is a target of hsa-miR-20b. Knockdown of hsa-miR-20b attenuated premature senescence in the TNF-α-treated HMVEC-Ls as evidenced by increased cell proliferation, increased RBL1 mRNA expression level but decreased protein expression of $p16^{INK4a}$, a cellular senescence marker. These findings provide an early insight into the role of hsa-miR-20b in endothelial senescence.



## 1. Introduction

Cellular senescence is a state of irreversible growth arrest first described following the observation of cells losing their proliferative capacity after a finite number of population doublings (Hayflick, 1965; Hayflick and Moorhead, 1961). Many types of stressors can induce cellular senescence, including oxidative stress, DNA damage, activation of tumor suppressor gene, improper cell contacts or chronic exposure to the pro-inflammatory cytokines as well as telomere shortening from repeated cell division, which is also known as replicative senescence (RS) (Ben-Porath and Weinberg, 2005; Foreman and Tang, 2003; Toussaint et al., 2001). Cytokine-induced premature senescence (CIPS) has been associated with persistent DNA damage response and activation of p53-$p21^{CIP1}$ and $p16^{INK4a}$ retinoblastoma protein (pRB) cell cycle regulatory pathways, which cause growth arrest and senescence (Sasaki et al., 2008). In addition, CIPS is often reinforced by a plethora of senescence-associated secretory phenotypes factors including tumor necrosis factor alpha (TNF-α), interleukins (IL-1α, IL-1β, IL-6), transforming growth factor beta (TGF-β) and chemokines such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (Kojima et al., 2013).

The pulmonary vasculature is lined by a monolayer of endothelial cells with a total surface area of 90 $m^2$ and it is critical for gas exchange in the lung. This monolayer of cells acts as a selective barrier by lying at the interface between blood, airway and lung parenchyma (Goldenberg and Kuebler, 2015). Premature senescence of pulmonary endothelial cells can induce endothelium dysfunction which could increase susceptibility to diseases such as pulmonary sepsis and pulmonary hypertension in elderly patients. Extensive studies have shown that aging pulmonary endothelium is more vulnerable to oxidative stress because of decreased antioxidant activities, reduced bioavailability of nitric oxide which is a vasorelaxant molecule (Rapoport et al., 1983) and insufficient pulmonary cellular repair and regeneration (Jane-Wit and Chun, 2012). In addition, endothelium dysfunction in lung has been reported to be one of the causes of exacerbation of lung inflammatory-

*Abbreviations:* RS, replicative senescence; CIPS, cytokine-induced premature senescence; pRB, retinoblastoma protein; TNF-α, tumor necrosis factor alpha; SA-β-gal, senescence-associated beta-galactosidase; HMVEC-Ls, human pulmonary microvascular endothelial cells; PD, population doublings; RBL1, retinoblastoma-like 1; Bi, biotin; HUVECs, human umbilical vein endothelial cells; RTCA, Real Time Cell Analyzer; CI, cell index; SEM, standard error of the mean; ANOVA, one-way analysis of variance.

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related disease such as asthma (Mukhopadhyay et al., 2006; Suarez et al., 2010) and chronic obstructive pulmonary disease (COPD) (Green and Turner, 2017). In situ culture of lung tissue collected from COPD patients showed hallmarks of senescence such as decreased cell-population doublings, early replicative senescence, shorter telomeres, higher levels of p16 and p21 and increased secretion of inflammatory markers at early cell passage compared to those in control subjects (Amsellem et al., 2011).

TNF-α is a pleiotropic pro-inflammatory cytokine of the TNF superfamily produced by various cell types and activated macrophages. TNF-α exerts multiple biological effects depending on the distinct receptors expressed on the specific cell types (van Horssen et al., 2006). Essentially, overexpression of TNF-α activates intracellular signaling cascades of apoptosis, cell survival, inflammation, immunity and senescence. An earlier study showed that TNF-α-treated human diploid fibroblasts exhibited increased senescence-associated beta-galactosidase (SA-β-gal) activity and irreversible growth arrest which confirmed that TNF-α could induce premature senescence (Dumont et al., 2000). Several other studies further demonstrated that this inflammatory stressor could trigger premature senescence in endothelial cells (Dumont et al., 2000; Zhao et al., 2010; Zhou et al., 2002) by generating reaction oxygen species (Dumont et al., 2000) or by altering mitochondrial functions (Zhou et al., 2002).

miRNAs are short (~22 nt), non-coding RNAs (Lee et al., 1993) which regulate gene expression by targeting the 3′-untranslated region (UTR) of mRNA transcripts, causing translation inhibition or degradation of mRNA. Unique alterations in miRNA expression were observed in various pathological conditions including aging-associated diseases (Frenzel et al., 2009). miRNAs have been implicated in modulating senescence in various cell types. For examples, miR-217 and miR-34a were shown to target Sirtuin1 while miR-146a is reported to target NADPH oxidase 4 in the modulation of senescence in endothelial cells (Ito et al., 2010; Menghini et al., 2009; Tabuchi et al., 2012; Vasa-Nicotera et al., 2011; Zhao et al., 2010). Despite extensive investigations onto the role of miRNAs in cellular senescence, the regulation of cellular senescence pathways modulated by miRNAs remains to be further defined. The present study sought to investigate the role of hsa-miR-20b in premature senescence induced by chronic exposure to TNF-α. Findings from the present study would contribute new insights into the in-depth mechanisms governing endothelial health and dysfunction.

## 2. Materials and methods

### 2.1. Cell culture

Human pulmonary microvascular endothelial cells (HMVEC-Ls; Lonza, MD, USA) were maintained in complete microvascular endothelial cell growth medium-2 at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. Population doublings (PD) was calculated as described (Cristofalo et al., 2000). Cells were seeded at a constant initial density of $5 \times 10^5$ cells per T75 flask and passaged using 0.25% trypsin EDTA every two to three days in order to maintain the cultures in constant log phase. The cells were used at passage 5 (young untreated cells), passage 12 (pre-senescent cells), or passage 19 (senescent cells), in subsequent experiments. HMVEC-Ls were used because they are more delicate in physical structure and thus, more susceptible to the induction of endothelial dysfunction.

### 2.2. Induction of replicative senescence (RS) in HMVEC-Ls

RS cells were obtained by consecutively passaging the young untreated cells until cell proliferation ceased, approximately at passage 19 (PD ~20). Pre-senescent cells were achieved by routine sub-culturing of the young untreated cells until passage 12 (PD ~13).

### 2.3. Induction of cytokine-induced premature senescence (CIPS) by TNF-α treatment

To establish CIPS model, young untreated cells of passage number <5 were subjected to chronic TNF-α (Sigma-Aldrich, USA) treatment at various concentrations. The cells were seeded and incubated overnight prior to fifteen successive sub-lethal treatments with or without TNF-α at concentrations of 10 ng/ml, 20 ng/ml and 40 ng/ml in complete media. The media were replenished every day for fifteen consecutive days.

### 2.4. SA-β-galactosidase (SA-β-gal) pH 6.0 staining

Senescent cells were detected by fluorescence-based SA-β-gal assay (Debacq-Chainiaux et al., 2009). Young and RS cells at passage <5 and >20, respectively, were trypsinized and seeded at a sub-confluent level overnight and pre-treated with 50 nM Bafilomycin A1 for 1 h. Similarly, at the end of the TNF-α chronic treatment, cells were trypsinized and subjected to the same procedure prior to incubation with Bafilomycin A1. $C_{12}$FDG, a fluorogenic substrate for β-galactosidase was then added to a final concentration of 33 μM and further incubated for 2 h. The cells were resuspended in ice-cold PBS with 5% FBS and acquired using FACSCanto II flow cytometer (Becton Dickinson, USA). The $C_{12}$FDG-fluorescein signal was detected by FITC detector channel. The percentage of senescent positive cells was determined using FlowJo Version 10 (Tree Star, USA).

### 2.5. Cell cycle analysis

Young, RS and CIPS (TNF-α-treated) cells were harvested and stained with propidium iodide using Cycletest plus DNA reagent kit (Becton Dickinson, CA, USA). DNA QC particles (Becton Dickinson) were used to verify the instrument performance and for quality control.

### 2.6. miRNA microarray

Agilent human microRNAs array consisting of 866 human miRNAs and 89 human viral miRNAs, based on Sanger miRNA database release 12.0 was used. Three biological replicates of RS and CIPS were used for microarray analysis. Total RNAs (100 ng) were labeled with Cyanine 3-pCp and hybridized onto Human MicroRNA Array version 3.0 (8X15K) with 40–60 mer oligonucleotides. Microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession no. GSE45541.

### 2.7. Microarray data analysis

miRNA microarray data was performed using GeneSpring GX version 11.5.1 (Agilent Technologies Inc., CA, USA). Threshold of raw signals were set at 1.0. The normalization algorithm employed was quantile and baseline transformation to median of all samples was performed. Differential miRNA profiles were generated by applying fold change >2.0 and corrected *P*-value < 0.05. Significance analysis was performed using Student's *t*-test followed by Benjamini-Hochberg multiple testing corrections. TargetScan program integrated into GeneSpring was used for the prediction of biological targets of the differentially expressed miRNAs.

### 2.8. qRT-PCR validation of miRNA and mRNA expression levels

miRNA and mRNA were harvested from cells by using miRNeasy Mini Kit (Qiagen, USA) and RNeasy Mini Kit (Qiagen, USA), respectively, following manufacturer's instructions. Total RNA yield was quantitated using NanoDrop 2000c (Thermo Scientific, USA). qRT-PCR was performed to quantitate the expression level of selected miRNAs using