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A Screen Identifies the Oncogenic Micro-RNA miR-378a-5p as a Negative Regulator of Oncogene-Induced Senescence

Susanne Marije Kooistra1,2, Lise Christine Rudkjær Nørgaard1,2, Michael James Lees1,2, Cornelia Steinhauer1,2, Jens Vilstrup Johansen1,3, Kristian Helin1,2,4*

1 Biotech Research and Innovation Centre, University of Copenhagen, Copenhagen, Denmark, 2 Centre for Epigenetics, University of Copenhagen, Copenhagen, Denmark, 3 The Bioinformatics Centre, Department of Biology, University of Copenhagen, Copenhagen, Denmark, 4 The Danish Stem Cell Center (DanStem), University of Copenhagen, Copenhagen, Denmark

Abstract

Oncogene-induced senescence (OIS) can occur in response to hyperactive oncogenic signals and is believed to be a fail-safe mechanism protecting against tumorigenesis. To identify new factors involved in OIS, we performed a screen for microRNAs that can overcome or inhibit OIS in human diploid fibroblasts. This screen led to the identification of miR-378a-5p and in addition several other miRNAs that have previously been shown to play a role in senescence. We show that ectopic expression of miR-378a-5p reduces the expression of several senescence markers, including p16INK4A and senescence-associated β-galactosidase. Moreover, cells with ectopic expression of miR-378a-5p retain proliferative capacity even in the presence of an activated Braf oncogene. Finally, we identified several miR-378a-5p targets in diploid fibroblasts that might explain the mechanism by which the microRNA can delay OIS. We speculate that miR-378a-5p might positively influence tumor formation by delaying OIS, which is consistent with a known pro-oncogenic function of this microRNA.

Introduction

Senescence is a permanent exit from the cell cycle that can be driven by different cellular and environmental signals. Critical shortening of telomeres, oxidative stress, DNA damage and aberrant activation of certain oncogenes can all lead to cellular senescence [1,2]. The latter case is termed oncogene induced senescence (OIS) and is thought to function as a mechanism to prevent tumorigenesis [3,4]. The presence of oncogenic BRAVOp530E, for example, results in senescence in vivo, which is evident from senescent melanocytes that can be found in benign human naevi [5].

Although the precise molecular pathway leading from the activated oncogenes to the final growth arrest is not known, it involves activation of the RAF-MEK-ERK kinase pathway that results in transcriptional activation of the INK4A-ARF-INK4B locus. This important tumor suppressor locus codes for 3 proteins (p16INK4A, p15INK4B and p14ARF) that are all activated by cellular stress and regulate cellular proliferation by feeding into the p53 and pRB tumor suppressor pathways [6]. Mouse cells depend on both p19ARF/p53 and p16INK4A/pRB pathways during OIS. However, in human cells it appears that p16INK4A plays a more prominent role, as certain cell types only require p16INK4A expression to induce senescence [7,8]. The importance of this locus for senescence is also evident from the fact that among the factors that have been identified to regulate OIS are several proteins that regulate expression of the INK4A-ARF-INK4B locus, including the Polycomb group protein BMI1 [9,10] and the histone demethylase JMJD3 [11,12].

Recent results have shown the importance of senescence as a tumor barrier in vivo. For instance, failure to clear senescent cells after introduction of oncogenic Nras in the liver leads to increased incidence of hepatocellular carcinoma [13]. The protective effect involves immune surveillance and extensive communication of the pre-malignant senescent cells with their environment, probably through the acquisition of the senescence-associated secretory phenotype (SASP) [13,14].

Several microRNAs (miRNAs) have also been shown to be involved in senescence. miRNAs are transcribed, processed into hairpin intermediates called pre-microRNAs (pre-miRs), and cleaved to give mature 21–23 nucleotide long miRNAs that often function by targeting the 3’ untranslated region (UTR) of mRNA transcripts, thereby downregulating the expression of their targets [15]. miRNAs that can either induce or help cells to evade senescence have been identified and they include miRNAs that target the p53 pathway, the p16INK4ApRB pathway and the SASP [16,17].

To identify new factors involved in OIS, we performed a screen for miRNAs that can overcome or inhibit OIS in human diploid fibroblasts, using p16INK4A expression as a readout. We expressed
a library of 471 human pre-miRs in human fibroblasts, which were subsequently induced to senesce by activation of the Braf oncogene [18]. Our screen identified several miRNAs that can regulate senescence, among which miR-378a-5p (previously miR-378 and miR-378a), a miRNA that is expressed in several types of cancer and has oncogenic properties [19–23]. The introduction of miR-378a-5p oligonucleotides resulted in reduced activation of p16INK4A upon activation of Braf and allowed cells to retain proliferative capacity even in the presence of the activated oncogene. We furthermore identified putative miR-378a-5p target miRNAs in human fibroblasts by high throughput RNA sequencing. Taken together our results suggest that miR-378a-5p can have a positive effect on tumor formation by preventing full activation of the senescence program. These results are in agreement with the oncogenic features of miR-378a-5p.

**Results**

**Identification of miRNAs regulating OIS**

To identify miRNAs with a role in OIS, we used the human diploid fibroblast line TIG3, which was immortalized with telomerase and expressed a conditional form of the mouse Braf oncogene (ΔBraf:ER), that allows senescence to be induced by treatment with 4-hydroxytamoxifen (4-OHT) [11,18]. Immunofluorescent staining of p16INK4A was used as a measure for senescence and the screen was essentially performed as follows: cells were transfected on day 0, treated with 1 μM 4-OHT on day 2 and fixed for analysis on day 4 (Figure 1a). A library containing 471 human pre-miRs (miRNA mimics) was reverse transfected into TIG3-hTERT-ΔBraf:ER cells and their effect on senescence determined (Figure 1b). In addition to a scrambled control (SCR), we used siRNAs against BMI1, INK4A and JMJD3 (BMI1i, p16i, JMJD3i) as controls in every plate in the screen. Cells transfected with the SCR control were treated with ethanol as a technical control for the staining and image analysis procedure (SCR (-) Figure 1). The behavior of the controls in each plate was determined (Figure S1a). The control siRNAs included in the screen (i.e. knockdown of BMI1 or JMJD3) have been shown to play a biological role in senescence [9–12] and therefore all miRNAs that performed better than the control siRNAs were considered potential hits (listed miRNAs in Figure 1b). In the screen we identified 16 miRNAs that had a positive effect on p16 expression and 7 miRNAs whose expression resulted in reduced p16 levels. Images of controls and selected miRNAs are shown in Figure 1c, pictures for all miRNAs considered hits are depicted in Figures S2, S3). The fold change in percentage p16 positive cells relative to each plate average was approximately 2.25 for hits increasing (Figure 1d) and 5–10 fold for hits decreasing (Figure 1e) after 24 and 48 hours. This was confirmed by BrdU incorporation in these cells. We found that miR-378a-5p transfected cells showed increased EdU incorporation, while at the same time p16INK4A levels were reduced upon Braf activation (Figure 2c) after 24 and 48 hours. This was confirmed by BrdU incorporation assay after 48 hours, where only 0.13% of the SCR control cells had incorporated BrdU vs 4.09% of the miR-378a-5p expressing cells (Figure 2f). Taken together these results show that miR-378a-5p expression can lead to a delay in execution of oncogene-induced senescence.

**Identification of potential miR-378-5p target mRNAs**

miRNA genes are transcribed and in the process of generating the mature miRNA, two distinct mature miRNAs can be formed and become active. We measured the expression of both mature miRNAs transcribed from the miR-378a gene, miR-378a-5p and miR-378a-3p, by RT-qPCR analysis and found that both are expressed at low levels in TIG3 cells (Figure 3a). We expected to find miR-378a-5p at low levels, as it counteracts senescence, which these cells can normally undergo upon receiving the appropriate signals. In order to determine whether the effect we observe for miR-378a-5p is specific to this miRNA, we expressed miR-378a-3p oligonucleotides in TIG3-hTERT-ΔBraf:ER cells, which did not result in decreased p16INK4A protein levels upon senescence induction (Figure 3b) as compared to SCR control transfected cells (Figure 2f). Two targets of miR-378-5p as predicted by Targetscan 5.2 [29], SPI1 and SFRP1, have been implicated in regulation of p16INK4A [30,31]. Therefore, we tested whether their expression was affected by miR-378a-5p in TIG3-hTERT-ΔBraf:ER cells, and if their knockdown was sufficient to copy the phenotype
Figure 1. Identification of miRNAs that affect p16<sup>INK4A</sup> expression during oncogene-induced senescence. (a) Graphical representation of the workflow. The screen was performed in 384 well-format in TIG3 ΔBraF<ER> cells with a library containing 471 human pre-miRs. 48 hours after transfection the cells were treated with 4-hydroxy-tamoxifen (4-OHT) for 48 hours and p16<sup>INK4A</sup> levels were determined by immunofluorescence (IF) and analyzed automatically. (b) Screen results. The percentage of p16<sup>INK4A</sup> positive cells was determined for each well using the Hoechst signal to determine the total cell number. Following automated image analysis, the Z-score was calculated for each miRNA based on the average percentage of p16<sup>INK4A</sup> positive cells per plate. All listed miRNAs are considered potential hits; the ones indicated in bold were chosen for further validation. BMI1i, p16i and JMJD3i represent controls in which BMI1, p16INK4A or JMJD3 were downregulated by siRNAs. SCR: scrambled control. (-) indicates scrambled control cells in which senescence was not induced. (c) Immunofluorescence images taken as part of the screening process. Controls and selected hits are shown. (d) Fold change relative to the plate average of all hits increasing the percentage of p16<sup>INK4A</sup> positive cells in the screen (red line indicates the overall average). (e) Fold change relative to the plate average of all hits decreasing the percentage of p16<sup>INK4A</sup> positive cells in the screen (red line indicates the overall average). (f) The average of p16<sup>INK4A</sup> induction in 5 independent experiments. IF for p16<sup>INK4A</sup> followed by automated image analysis was used as a readout and scrambled (SCR) control samples were used for normalization. Averages are shown with SEM.
and t-tests were used to evaluate differences. Asterisks indicate a significant difference (p<0.001) in p16\(^{INK4A}\) induction.
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observed upon miR-378a-5p over expression. However, we did not find the two genes differentially expressed and knockdown of SUFU or SP1 alone using 2 different siRNAs per gene or in combination did not result in altered expression of p16\(^{INK4A}\) in response to Braf expression in TIG3 cells (Figure S4a, b). Therefore, the two genes did not explain the effect of miR-378a-5p, and we used RNA sequencing to identify genes whose expression was altered after expressing miR-378a-5p.

Cells were transfected with miR-378a-5p oligonucleotides or controls, and mRNA was extracted 48 hours after transfection. mRNA of biological replicate samples were sequenced and analyzed using TopHat and Cuffdiff [32]. We identified 309

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**Figure 2.** miR-378a-5p overexpression impairs oncogene-induced senescence. TIG3 ΔBrafER cells were transfected with scrambled control (SCR) or miR-378a-5p or miR378a-3p and after 48 hours treated with 4-OHT (+) or ethanol (−). (a) RT-qPCR using the housekeeping gene RPLPO as a reference and (b) western blot analysis of p16\(^{INK4A}\) expression after 24 and 48 hours of senescence induction (c) Senescence-associated β-gal staining after 48 hours of ethanol or 4-OHT treatment. (d) RT-qPCR analysis of the indicated senescence markers after 24 and 48 hours. Expression of RPLPO was determined and used for normalization. (e) Quantification of EdU and p16\(^{INK4A}\) staining of cells treated with ethanol or 4-OHT for 24 and 48 hours. Averages of 3 replicates are shown with the standard deviation. (f) Flow cytometric analysis of BrdU and propidium iodide staining of cells treated with ethanol or 4-OHT for 48 hours.
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genes that were upregulated (Figure 3c, table S1) and 227 genes that were downregulated in response to miR-378a-5p expression (Figure 3d, table S1). These differences in expression comprise both primary and secondary effects. As miRNAs target their mRNAs using a 6–8 nucleotide long seed sequence present in their 5′ prime end, we overlapped the list of differentially expressed genes with the predicted targets for miR-378a-5p (Figure 3e). Downregulated genes containing a match for the seed-sequence of miR-378a-5p with their relative fold change and false discovery rates (q-values) are indicated in Figure 3e. We then validated the expression changes in 3 independent biological replicates (Figure S5a) and could confirm the downregulation by miR-378a-5p of 20 genes (GNPDA1, STAMBPA, HNRNPA3, KLF9, PCBP2, TMUB2, SLC7A11, LIMD1, RSAD1, TRIM44, KPNA1, VGLL3, HOOK3, LATS2, KLF13, NDEL1, FOXN3, PLAU, ZZZ3 and MCFD2).

Though several of these have been described as potential tumor suppressor genes, or to be downregulated in different tumor types [33–42], the role of these proteins in senescence induction has not been determined.

In addition to the targets that we identified by RNA sequencing, miR-378a-5p has been shown to be involved in tumorigenesis and tumor maintenance by regulating SUFU, TUSC2, TOB2, GABPA and ESRRg [19–23]. In our RNA sequencing experiment, the expression of these genes was not significantly altered and we therefore analyzed their expression by RT-qPCR (Figure S5b). Here again we found that they were not differentially expressed upon miR-378a-5p expression, and it is therefore unlikely that these genes contribute to regulating senescence in TIG3-hTERT-ΔBraf:ER.

**Discussion**

The microRNA miR-378a-5p previously has been identified as an oncogene, where it has been shown to function in different pathways depending on the cell type studied. miR-378a-5p enhances cell survival, proliferation rate and angiogenesis in glioblastoma cells, through targeting of SUFU and TUSC2 [19]. In mammary cells, miR-378a is a transcriptional target of Myc, and it regulates oncogenic transformation through the regulation of TOB2 expression [20]. In addition, miR-378a-5p has been shown to be involved in increasing cell proliferation of breast cancer cells by mediating a metabolic shift. Through downregulation of GABPA and ERRγ, two PGC-1b partners, miR-378a-5p helps to orchestrate the Warburg effect in breast cancer cells [21].

Here, we have shown that over expression of miR-378a-5p allows human fibroblasts to escape the full senescence program upon induction of oncogenic Brf. As the expression of none of the previously published targets of miR-378a-5p is affected in TIG3 cells, they are most likely not involved here. Therefore we performed experiments to identify 20 potential mRNA targets of miR-378a-5p in human fibroblasts. Even though further experiments will be required to determine the contribution of the 20 identified target genes in oncogene-induced senescence, we hypothesize that one, or perhaps more likely, more than one of these 20 putative miR-378a-5p target genes contribute to the observed phenotype. In summary, we have shown that the oncogenic microRNA miR-378a-5p can contribute to overriding oncogene-induced senescence in vitro. Since senescence forms a barrier against tumor formation in vivo, we speculate that the
observed effect on senescence induction could provide miR-378a-5p with an additional mechanism for how it is involved in tumorigenesis.

**Materials and Methods**

**Cell culture and siRNA transfections**

The human diploid cell line TIG3 (from the Japanese Cancer Research Resources Bank, Tokyo, Japan) was immortalized with telomerase (hTERT) and transduced with a retrovirus generated from pMSCVpuro-ΔBraf:ER in order to be able to induce senescence [11,13]. Cells were maintained in DMEM (Gibco) supplemented with 10% FBS (Hyclone) and penicillin/streptomycin (Gibco). Senescence was induced by treatment with 1 μM 4-hydroxytamoxifen (4-OHT, Sigma) after which they were permeabilized with Triton-x-100 and stained by adding an equal volume of 4% formaldehyde to the wells, after with control siRNAs (scrambled, JMJD3i, BMI1i). Cells were fixed in (Gibco). Senescence was induced by treated with 1 μM 4-hydroxytamoxifen (4-OHT, Sigma) from a 1 mM stock in 96% ethanol. As a control, cells were treated with ethanol alone.

siRNA and miRNA oligonucleotides were introduced into TIG3 cells at a final concentration of 50 nM by reverse transfection using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The siRNAs and miRNA oligonucleotides that were used are listed in table S2.

**siRNA screen and p16INK4A immunofluorescence**

The screen was performed using a human-pre-miR-library containing 471 human pre-miRs (Ambion). TIG3-hTERT-ΔBraf:ER cells were reverse transfected in quadruplicates in 384-well plates and after 48 hours treated with 1 μM of 4-OHT for an additional 48 hours. Each plate contained cells transfected with control siRNAs (scrambled, JMJD3i, BMI1i). Cells were fixed by adding an equal volume of 4% formaldehyde to the wells, after which they were permeabilized with Triton-x-100 and stained with p16INK4A antibody (sc-56330, Santa Cruz) and Hoechst 333258 (Invitrogen). Automated image analysis was performed on the INCell Analyzer 1000 (GE Healthcare) using a 20× objective and 6 images per well (containing approximately 100 cells per image) and the percentage of p16INK4A positive cells per image determined using the INCell Analyzer Workstation 3.6 software (GE Healthcare). The Hoechst signal was used to determine the total cell number in each image and the p16INK4A values of the 6 images were averaged for further analysis. For hit determination, Z-scores were calculated for each well using the respective plate images (TIF) and the percentage of p16INK4A positive cells per image

**Antibodies for Western blotting**

The following antibodies were used in Western blot analysis: p16ink4A (DCS50), ΔBraf:ER (sc-166 Santa Cruz) and β-actin (Ab6276 Abcam)

**Senescence associated β-galactosidase staining**

Senescence associated β-galactosidase staining was performed as previously described [43].

**BrdU and EdU incorporation assays**

Cells were pulsed with 33 μM BrdU or 8 μM EdU for 3 hours prior to fixation. BrdU treated cells were fixed and stained with an antibody against BrdU (Beckon & Dickinson) and propidium iodide (Sigma), after which they were analyzed by flow cytometry using a FACSCalibur (BD biosciences). EdU treated cells, which were grown in 96-well plates, were fixed and stained for p16INK4A as described above. EdU was detected using Click-IT EdU chemistry (Invitrogen) according to manufacturer’s protocol and automated image analysis as described above.

**RNA extraction and RT-qPCR**

RNA for mRNA expression analysis was extracted using the RNeasy Plus kit (Qiagen) and reverse transcribed using Taqman reverse transcription reagents (Applied Biosystems). Quantitative PCR was done on a LightCycler480 (Roche), using LightCycler 480 SYBR Green I Master mix (Roche). Primer sequences are listed in table S3. RNA for miRNA expression analysis was extracted with the miRNeasy kit (Qiagen), reverse transcribed with Taqman MicroRNA reverse transcription kit (Applied biosystems) and quantified using Taqman MicroRNA assays for miR-191 (assay ID 002299), miR-378-3p (assay ID 002243) and miR-378-5p (assay ID 000567). Differences in expression were determined using the 2-DDCt method [44], using the housekeeping gene RPLP0 and miR-191 for normalization.

**RNA sequencing**

TIG3-hTERT-ΔBraf:ER cells were transfected with scrambled control or miR-378-5p oligonucleotides. 48 hours after transfection, RNA was extracted from 3 biological replicates, it’s quality monitored on the 2100 expert Bioanalyzer (Agilent), and prepared for sequencing using the NEBNext mRNA sample Prep Master Mix Set I (New England Biolabs). The amplified cDNA was analyzed by Solexa/Illumina high-throughput sequencing. The tags were mapped to the human genome (assembly hg19) with TopHat [45] and differential expression was determined with Cufflinks [32] at an FDR cut-off value <0.5.

**Supporting Information**

- **Figure S1** Identification of miRNAs that affect p16INK4A expression during oncogene-induced senescence. (TIF)
- **Figure S2** p16INK4A induction during the screen. (TIF)
- **Figure S3** p16INK4A induction during the screen. (TIF)
- **Figure S4** SUFU and SPI1 are not involved in regulation of senescence by miR-378a-5p. (TIF)
- **Figure S5** Validation of RNA sequencing. (TIF)
- **Table S1** RNA sequencing data. (XLS)
- **Table S2** Sequences of siRNA and miRNA oligonucleotides. (DOCX)
- **Table S3** Primers used for RT-qPCR analysis. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: SMK KH. Performed the experiments: SMK LCRN ML CS. Analyzed the data: SMK LCRN ML CS JJ KH. Contributed reagents/materials/analysis tools: SMK LCRN ML CS JJ KH. Wrote the paper: SMK KH.
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