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MicroRNA transcriptome analysis identifies miR-365 as a novel negative regulator of cell proliferation in *Zmpste24*-deficient mouse embryonic fibroblasts

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Abstract

Zmpste24 is a metalloproteinase responsible for the posttranslational processing and cleavage of prelamin A into mature laminA. *Zmpste24*^{-/-} mice display a range of progeroid phenotypes overlapping with mice expressing progerin, an altered version of lamin A associated with Hutchinson-Gilford progeria syndrome (HGPS). Increasing evidence has demonstrated that miRNAs contribute to the regulation of normal aging process, but their roles in progeroid

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disorders remain poorly understood. Here we report the miRNA transcriptomes of mouse embryonic fibroblasts (MEFs) established from wild type (WT) and *Zmpste24*^{-/-} progeroid mice using a massively parallel sequencing technology. With data from 19.5×10^6 reads from WT MEFs and 16.5×10^6 reads from *Zmpste24*^{-/-} MEFs, we discovered a total of 306 known miRNAs expressed in MEFs with a wide dynamic range of read counts ranging from 10 to over 1 million. A total of 8 miRNAs were found to be significantly down-regulated, with only 2 miRNAs upregulated, in *Zmpste24*^{-/-} MEFs as compared to WT MEFs. Functional studies revealed that miR-365, a significantly down-regulated miRNA in *Zmpste24*^{-/-} MEFs, modulates cellular growth phenotypes in MEFs. Overexpression of miR-365 in *Zmpste24*^{-/-} MEFs increased cellular proliferation and decreased the percentage of SA- β -gal-positive cells, while inhibition of miR-365 function led to an increase of SA- β -gal-positive cells in WT MEFs. Furthermore, we identified Rasd1, a member of the Ras superfamily of small GTPases, as a functional target of miR-365. While expression of miR-365 suppressed Rasd1 3'UTR luciferase-reporter activity, this effect was lost with mutations in the putative 3'UTR target-site. Consistently, expression levels of miR-365 were found to inversely correlate with endogenous Rasd1 levels. These findings suggest that miR-365 is down-regulated in *Zmpste24*^{-/-} MEFs and acts as a novel negative regulator of Rasd1. Our comprehensive miRNA data provide a resource to study gene regulatory networks in MEFs.

Keywords

miRNA; *Zmpste24*; miR-365; premature senescence; HGPS

1. Introduction

A-type lamins consist of two predominant (lamins A and C) and other isoforms that are generated through alternate processing of the *LMNA* gene. They are type V intermediate filaments and core constituents in the nuclear lamina. Lamin A is initially synthesized as a precursor, prelamin A, which undergoes a series of -CAAX box dependent processing events including isoprenylation at the cysteine residue and two proteolytic cleavage events leading to a mature lamin A reduced in size by 18 residues due to an internal cleavage event. The terminal proteolytic cleavage event is mediated by *Zmpste24* (also called FACE-1 in humans) [1]. A *de novo* mutation at G608G position promotes altered splicing of lamin A leading to the mutant protein progerin, which lacks an internal stretch of 50 amino acids including the *Zmpste24* cleavage site but retains the isoprenylated cysteine residue. Expression of this form of lamin A, termed progerin, is associated with Hutchinson-Gilford progeria syndrome (HGPS)[2-5].

Several mouse models of HGPS, most involving expression of progerin, have been generated and they exhibit variable phenotypes that overlap with those of HGPS patients[6]. Aspects of the HGPS phenotype are also recapitulated in mice deficient for the prelamin A processing enzyme *Zmpste24*, including hair loss, growth retardation, osteoporosis, and reduced lifespan[7-10]. Moreover, these phenotypes can be diminished by loss of one copy of lamin A, indicating that they arise due to expression of an unprocessed form of prelamin A[11]. Therefore, *Zmpste24*^{-/-} progeroid mice are considered to be a valuable tool in the study of the pathological processes implicated in the origin of HGPS[8]. However, although

aberrant lamin A leads to pathologies resembling premature aging in both humans and mice, the underlying mechanisms are still largely unknown[12].

MicroRNAs (miRNAs) are a class of endogenous, small, noncoding, single-stranded RNAs of approximately 22 nucleotides encoded within the genome and derived from endogenous short hairpin precursors. In mammals, miRNAs guide proteins of the argonaute family to partially-complementary sequences typically located in the 3'UTR of specific target mRNAs, leading to translational repression or mRNA degradation[13]. As the recognition of target mRNAs mainly depends on the small seed region within the mature miRNA, a single miRNA potentially regulates up to several hundred mRNA targets, thus orchestrating a large variety of biological functions. Growing evidence has suggested that they are involved in the control of a wide range of physiological pathways, such as development, differentiation, growth, and metabolism, as well as in disease conditions[14-16]. Recent studies have identified roles for miRNA in modulating the normal aging process [17-22]. However, very little is known about the importance of miRNAs in progeroid disorders[23]. In the current study, the alterations in the miRNA profiles of MEFs isolated from WT and *Zmpste24*^{-/-} progeroid mice were investigated. Further analyses revealed that miR-365, one of the dysregulated miRNAs in *Zmpste24*^{-/-} MEFs, regulates cellular proliferation and premature senescence in part through its target gene, Rasd1.

2. Materials and methods

2.1 Cells and Cell Culture

Zmpste24^{-/-} and WT MEFs, and mouse myoblast cell line C2C12 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum. Serial passage (P) was performed when the cells reached an 80% confluence.

2.2 RNA preparation

Total RNA was extracted from *Zmpste24*^{-/-} and WT MEFs at passage 3 using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quality and quantity of the RNAs were assessed by A260/A280 nm reading using NanoDrop1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was determined by running an aliquot of the RNA samples on a denaturing agarose gel stained with SYBR Green I.

2.3 Small RNAs enrichment

Small RNA enrichment from total RNAs was performed by following the manufacturer's instructions (mirVana miRNA isolation kit, Ambion). 1/3 volume of 100% ethanol was added to the aqueous phase recovered from the organic extraction and centrifuged. Another 2/3 volume of 100% ethanol was added and mixed thoroughly. After centrifugation, the filter cartridge was washed by miRNA wash solution 1 and 2/3 and then RNA was eluted with 95°C elution solution.

2.4 cDNA library construction and deep sequencing

MiRNA-seq was performed as indicated by the manufacturer (Illumina small RNA prep kit v 1.5, San Diego, CA, USA). 2µg of total RNA from each sample was ligated with a biotinylated RNA-DNA 3'-adaptor, and then ligated with a 5'-adaptor. Small RNAs were resolved on a 15% TBE-Urea polyacrylamide gel followed by the excision of gels corresponding to the 17-35 nucleotides and isolated from the gel in 300µl of 0.3M NaCl for 12 hours at room temperature. The small RNA products with both adaptors were reverse-transcribed and PCR amplified for 12 cycles. Amplified DNAs were selected by the excision on 10% TBE polyacrylamide gel and purified. Sequencing was performed on an Illumina GA1 analyzer.

2.5 Data analysis

The sequencing data was provided from the GA1 sequencer in a standard fastq format [24]. The fastq files were trimmed of adapter sequences and of low quality reads (reads which had more than 3 base-calls below sufficient quality value), through a c++ program. These sequences were then collapsed to remove redundancy using the Galaxy Genome Browser tool fastx[25]. At this point sequences from each of the samples were aligned to the known human miRNA/small RNA database or put into the mirDeep pipeline for the discovery of novel miRNA. All of the putative miRNAs and their genomic locations have been submitted to MirBase. The miRNA tags matched were statistically normalized on a tags (determined miRNA) per total read (result from sequencer) basis[26]. The basis of this idea is that for a given number of small sequences isolated from a cell there should be on average the same number of total miRNA from the sample. After normalization any reads that were in fewer than 3 samples and in copy number less than 10 were not considered for analysis, this correction removed extremely low abundance miRNA. Finally the data was analyzed through Fisher's exact test using a Bonferonni correction for multiple hypothesis testing. Those miRNAs meeting a corrected cutoff with a p-value below 0.05 and with a fold change greater than 2.0 were considered differentially expressed (Table 1).

MicroRNA targets were predicted by these databases: TargetScan (http://www.targetscan.org/mmu_50/), miRDB (<http://mirdb.org/miRDB/>), MicroCosm (<http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl>), PicTar (<http://pictar.mdc-berlin.de/>) and miRanda (<http://www.microrna.org/microrna/getMirnaForm.do>).

2.6 Real-time quantitative RT-PCR

For mRNA detection, total RNA was reverse transcribed using PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Japan). Real time PCR was performed in an ABI PRISM 7500 Sequence Detection System (Applied Biosystem) with SYBR green master mix (Takara). PCR primer sequences for Rasd1 were: Rasd1 Fwd (5'-GCCAGGCTAAGGACAAGG-3') and Rasd1 Rev (5'-TCAGGGAGGGAAGGAGAA-3'). PCR primer sequences for β -actin were: β -actin Fwd (5'-AGATGACCCAGATCATGTTTGAG-3') and β -actin Rev (5'-AGGGCATACCCCTCGTAGAT-3'). β -actin gene was used as internal control. For miRNA detection, RNA was reverse transcribed using miRCURY LNATM Universal cDNA Synthesis Kit and real time PCR was performed with SYBR[®] Green master mix (Exiqon, Vedbaek, Denmark) and specific primers for miRNAs (Exiqon). Expression levels were

normalized to U6 snRNA (Exiqon). Fold changes of mRNA and miRNAs were calculated by the 2^{-CT} method, after normalization against the expression of β -actin or U6 snRNA, respectively.

2.7 miRNA transfection

The day before transfection, *Zmpste24*^{-/-} and WT MEFs at early passage (passage 2-3) were plated in growth medium at a density of ~60-70% confluency. Transfection of miRNA mimic (Qiagen, Valencia, CA, USA) or miRNA inhibitor (Qiagen) was performed using HiPerFect Transfection Reagent (Qiagen). 10 nM of miRNA mimics and 50 nM of miRNA inhibitor were used for each transfection.

2.8 Senescence-associated β -galactosidase staining

Transfected cells were fixed, and stained with X-gal using staining kit provided from Sigma-Aldrich Co. (Louis, MO, USA) according to the manufacturer's instructions. The percentage of senescence-associated β -gal positive cells was determined by counting the number of blue cells within a sample of more than 800 cells.

2.9 Cell proliferation assay

Cell proliferation was analyzed by measuring DNA synthesis with the EdU Cell Proliferation Assay Kit (Ribobio, Guangzhou, China) according to the manufacturer's instructions. Briefly, *Zmpste24*^{-/-} MEFs (4×10^4 cells per well) were cultured in triplicate in 24-well plates and transfected with 10 nM of miRNA mimics for 72 h. Then cells were incubated with 50 nM of 5-ethynyl-2'-deoxyuridine (EdU) for an additional 2 h at 37°C. Cells were fixed with 4% formaldehyde for 30 min at room temperature and treated with 0.5% Triton X-100 for 10 min at room temperature to permeabilize cells. After washing with PBS three times, cells were incubated with 1× Apollo reaction cocktail for 30 min. DNA was stained with 10 μ g/ml of Hoechst 33342 for 30 min and visualized under a fluorescent microscope.

2.10 Vector Construction

A wild-type 3'-UTR segment of mouse Rasd1 (493 bp) mRNA that contained putative binding site for miR-365 was PCR-amplified and inserted into the *EcoR* I/*Xba* I site downstream of the stop codon of firefly luciferase in pGL3cm, which was created based upon the pGL3-control (Promega) and gifted from prof. Shi-mei Zhuang[27]. The resulting plasmid was denoted pGL3cm-Rasd1-WT-3'-UTR. The plasmid pGL3cm-Rasd1-MUT-3'-UTR, which carried mutated sequence in the complementary site for the seed region of miR-365 (Fig. 3C), was generated by site-specific mutagenesis. The sequences of all constructs were verified by direct DNA sequencing.

2.11 Luciferase reporter assay

C2C12 cells plated in a 48-well plate were co-transfected with 5 nM miRNA mimic, 20 ng pGL3cm-Rasd1-WT-3'-UTR or pGL3cm-Rasd1-MUT-3'-UTR, and 4ng of pRL-TK (Promega). Cells were collected 48 h after transfection and analyzed using Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was detected by an FB12

Luminometer (Titertek-Berthold, Germany). The pRL-TK vector that provided the constitutive expression of *Renilla* luciferase was used as an internal control to correct the differences in both transfection and harvest efficiencies. Transfections were done in duplicates and repeated three times in independent experiments.

2.12 Statistical analysis

Data were analyzed using GraphPad prism (GraphPad Software, Inc., San Diego, CA, USA). Statistical differences were determined by the student's *t*-test, with values of $P < 0.05$ considered statistically significant.

2.13 Accession number

The sequence data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under accession no. GSE46379.

3. Results

3.1 Discovery of miRNAs expressed in *Zmpste24*^{-/-} MEFs

We generated small RNA libraries of *Zmpste24*^{-/-} MEFs and littermate wild type controls. Sequencing of these libraries by an Illumina technology platform yielded a total of 19.5×10^6 reads from *Zmpste24* wild type MEFs and 16.5×10^6 reads from *Zmpste24*^{-/-} MEFs (Supplementary Table S1). After removal of low quality reads and redundancy, we had a total of 19.1×10^6 and 14.9×10^6 unique reads for WT and *Zmpste24*^{-/-} MEFs, respectively. Of the unique reads, those with a read number greater than 10 in more than 50% sequenced were aligned to a database of known miRNAs (<http://www.mirbase.org/>) and other known small RNA species. MiRNAs with less than 10 reads were not included due to the error rate of Illumina sequencing and stochastic variation in gene expression[28]. We identified a total of 306 known miRNAs (Supplementary Table S1) with a wide dynamic range of read counts ranging from 10 to over 1 million (Supplementary Table S2). About 24% of these miRNAs had a copy number greater than 10,000 and about 54% a copy number less than 1,000. The distribution of miRNA calls to total reads is similar to other reported findings from Illumina sequencing[28,29]. The percent normalized read count for each miRNA indicated that miR-143 (>30% total) was the most abundant miRNA followed by miR-21 (>20% total) detected in both WT and *Zmpste24*^{-/-} MEFs (Fig. 1). The top 10 miRNAs by read count corresponded to ~80% of all sequence reads in the WT and *Zmpste24*^{-/-} MEFs (Fig. 1). A complete list of miRNAs sequenced in each library, total and normalized read counts, and fold differences between WT and *Zmpste24*^{-/-} MEFs is provided in Supplementary Table S2.

We identified a total of 10 differentially expressed known miRNAs with a fold change ≥ 2.0 (Table 1) after adjusting for multiple testing with Bonferroni correction at a cutoff < 0.05 [30-32]. Of the 10 differentially expressed miRNAs, 2 were upregulated and 8 downregulated in *Zmpste24*^{-/-} MEFs as compared to controls (Table 1). Read counts for the differentially expressed miRNAs ranged from the 60s to over 40,000.

3.2 Targets of differentially expressed miRNAs

Among the 10 differentially expressed miRNAs, several have been previously characterized with known validated targets (Supplementary Table S3), including let-7c. Target sites of the 10 differentially expressed miRNAs were redacted using available computation approaches, in particular TargetScan (<http://www.targetscan.org>)[33], miRanda (<http://www.microrna.org/microrna/home.do>)[34], and PicTar (<http://pictar.mdc-berlin.de/>)[35]. These software packages work by finding an absolute base pair homology of the miRNA seed region (bases 2-8 on the 5' end of the miRNA) to the 3' UTR of all mRNA. In addition, they take into consideration evolutionary conservation of 3' UTR bases as well as RNA accessibility to the RISC complex. Due to the large number of predicted targets we only considered target genes with 2 predicted sites, an accessible 3'UTR and consensus amongst all prediction algorithms[29].

Based on this approach we identified predicted target genes for 10 differentially expressed miRNAs (Supplementary Table S3). To explore the potential pathways regulated by these miRNAs, we conducted Gene Ontology analysis utilizing the “GoStat” analysis tool (<http://gostat.wehi.edu.au/>). We took the total list of predicted and validated targets of the differentially expressed miRNAs and determined if any Gene Ontology categories were overrepresented within our list[36]. We found the enrichment of functional pathways implicated in the aging process, including cell metabolism, cell cycle, cell signaling, cell differentiation, and gene expression (Table 2) among other significant GO categories (Supplementary Table S4).

3.3 Biological validation of differential miRNA expression

We conducted quantitative real time PCR (qRT-PCR) analysis to validate the differential expression of miRNAs. Among 10 miRNAs found to be significantly different by Illumina sequencing analysis with fold change ≥ 2 in MEFs from *Zmpste24*^{-/-} as compared that from wild type mice (Table 1 and 2), optimized qRT-PCR kits for 7 miRNAs were available. qRT-PCR analysis is subject to certain limitations such as difficulties in detecting low abundance miRNAs and a limited dynamic range, essentially constraining the detection of subtle fold changes[28]. Nevertheless, two miRNAs, miR-342-5p and miR-365, were confirmed to be significantly down-regulated in *Zmpste24*^{-/-} MEFs (n=3) as compared to wild type MEFs (n=3) (Fig. 2).

3.4 MiR-365, a down-regulated miRNA in *Zmpste24*^{-/-} MEFs, influence a cellular proliferation and senescence phenotypes in MEFs

MiR-365 has been shown to be down-regulated during replicative senescence in lung fibroblast cells [37, 38] and expression of miR-365 has also been shown to be increased in cutaneous squamous cell carcinoma [39] and endometriosis [40], implicating its role in cell proliferation and senescence. To investigate the potential role of miR-365 on cell growth related phenotypes including the premature senescence observed in *Zmpste24*^{-/-} MEFs, we modulated miR-365 expression levels in both WT and *Zmpste24*^{-/-} MEFs. Transfection of a miR-365 mimic into *Zmpste24*^{-/-} MEFs led to 783±5 fold increase in miR-365 expression levels, while transfection of miR-365 inhibitor into WT MEFs decreased miR-365 levels to 9.1% of control (Fig. 3A). We found that reduction of miR-365 expression levels in WT

MEFs resulted in a significant increase in the number of senescent cells as measured by SA- β -gal activity at 6 days after transfection of miR-365 inhibitor (27.0% in miR-365 inhibitor transfected cells vs. 15.4% in negative control cells, Fig. 3B and C). Conversely, miR-365 overexpression in *Zmpste24*^{-/-} MEFs decreased the percentage of SA- β -gal-positive cells by 38% compared with control cells (Fig. 3B and C).

We next investigated whether miR-365 regulates cell proliferation in MEFs. We performed the EdU cell proliferation assay after the overexpression of miR-365 mimics in *Zmpste24*^{-/-} MEFs. We observed an increase in the incorporation of EdU in miR-365-overexpressed *Zmpste24*^{-/-} MEFs compared to controls (Fig. 4A). Quantification showed a significant increase of EdU-positive cells in miR-365 overexpressed *Zmpste24*^{-/-} MEFs indicating miR-365 promotes cell proliferation of *Zmpste24*^{-/-} MEFs (Fig. 4B). In contrast, down-regulation of miR-365 in wild type MEFs did not affect cell proliferation (data not shown). Taken together, these results suggest that cell proliferation phenotypes can be modulated by miR-365 expression levels in MEFs, raising the possibility that down-regulation of miR-365 in *Zmpste24*^{-/-} MEFs may contribute to premature senescence observed in these cells.

3.5 Rasd1 is a direct target of miR-365

The identification of miRNA-regulated gene targets is necessary for understanding miRNA functions. We used four miRNA target prediction programs that consider complementarity, target site accessibility, and the extent of evolutionary conservation (TargetScan, miRDB, MicroCosm and miRanda). All of these programs predicted that Rasd1 is a potential target of miR-365, suggesting that a putative binding site for the seed region of miR-365 is located at 343 to 349 bp of the mouse Rasd1 3' UTR (Fig. 5A). Although 3'UTR sequences tend to drift more rapidly than coding RNA sequences during evolution, the putative binding site for miR-365 in the Rasd1 3'UTR is highly conserved among different species (Fig 5A), suggesting an evolutionarily conserved miRNA-mRNA interaction with potentially important regulatory functions. The minimum free energy (MFE) of hybridization between the target RNA and miR-365, as predicted by RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>)[41] also supported the possibility that miR-365 can bind at that site (Fig. 5B).

To investigate whether Rasd1 is a *bona fide* target of miR-365, a mouse Rasd1 3'-UTR fragment containing wild-type or mutant miR-365-binding sequence (Fig. 5C) was cloned downstream of the firefly luciferase reporter gene. The relative luciferase activity of the reporter that contained wild-type 3'-UTR was significantly suppressed when miR-365 mimic was cotransfected into C2C12 cells (Fig. 5D). In contrast, the luciferase activity of mutant reporter was not affected by simultaneous transfection of miR-365 mimic (Fig. 5D). These results indicate that miR-365 can directly target the 3'UTR sequences of Rasd1. We also performed 3'UTR-reporter assays for other predicted targets of miR-365, including Pten and Sgk1 (Fig. 5E). However, there was no effect on the relative luciferase activity of the reporter harboring the wild-type 3'-UTR of Pten or Sgk1 when miR-365 mimic was co-transfected, suggesting that these genes are not targets of miR-365.

The effect of miR-365 on the endogenous expression of Rasd1 was further examined. We noted a trend toward higher expression of Rasd1 mRNA in *Zmpste24*^{-/-} MEFs compared to

WT MEFs, although the difference did not reach statistical significance at the $p = 0.05$ level (Fig. 6A). Knockdown of endogenous miR-365 significantly increased *Rasd1* mRNA level in WT MEFs (Fig. 6B). Consistently, overexpression of miR-365 significantly reduced *Rasd1* mRNA level in *Zmpste24*^{-/-} MEFs (Fig. 6B). Taken together, these results suggest that *Rasd1* is a molecular target of miR-365.

4. Discussion

Aging is a natural process that affects most biological functions and increases susceptibility to disease and death. Over the last few years, our knowledge of the molecular basis of aging has gained mechanistic insight from studies on progeroid syndromes in which features of human aging are manifested precociously or in an exacerbated form [2-6, 42]. *Zmpste24*^{-/-} mice exhibit multiple defects that phenocopy Hutchinson–Gilford progeria syndrome [7, 8]. Recent studies indicate that delineating the possible mechanisms underlying progeroid syndromes will be helpful to our understanding of the molecular mechanisms of physiological aging [43-45]. However, little is known about the role of miRNAs in progeria.

We employed a massively parallel sequencing technology to identify miRNAs expressed in WT and *Zmpste24*^{-/-} MEFs. This represents one of the first comprehensive studies to analyze miRNAs in progeroid cells. From this analysis, we obtained 36 million reads from WT and *Zmpste24*^{-/-} MEFs, resulting in the identification of 306 miRNAs. Profiling miRNA transcriptomes has gained importance with increasing evidence for the role of miRNA expression in defining cellular phenotypes. Prior to the advent, and subsequent cost reduction, of small RNA sequencing, microarrays have been the prevailing method for miRNA expression analysis. However, apart from noise due to cross-hybridization to probes with similar short-sequences, array technologies are limited in several key ways. First, microarrays are limited to the generated probe sets, eliminating the possibility to discover novel miRNAs. Second, arrays are only able to identify relative abundance of miRNA but not absolute count [26]. Sequencing allows accurate detection of expression levels over a wide dynamic range, including low copy number miRNAs and subtle fold changes between test and control groups.

Our analysis was aimed at identifying miRNAs that differentially expressed in *Zmpste24*^{-/-} MEFs relative to MEFs from littermate controls. We focused on miR-365 because the expression of miR-365 is markedly down-regulated in *Zmpste24*^{-/-} MEFs. Functional analysis revealed that miR-365 is a direct negative regulator of *Rasd1*. Importantly, we also found that miR-365 can modulate cellular growth phenotypes in MEFs. Overexpression of miR-365 in *Zmpste24*^{-/-} MEFs decreased the percentage of SA- β -gal-positive cells and increased cellular proliferation. In contrast, inhibition of miR-365 function in WT MEFs resulted in an increase of SA- β -gal-positive cells. These results suggest that the altered abundance of miR-365 in *Zmpste24*^{-/-} MEFs may not be a correlative finding, but instead may provide a causal link to premature senescence observed in these cells. This idea is supported by the observation that miR-365, is down-regulated during replicative senescence in lung fibroblast cells [37, 38]. Expression of miR-365 has also been shown to be increased in cutaneous squamous cell carcinoma [39] and endometriosis [40] and

downregulated when the potential tumor suppressor NGX6 is overexpressed in HT-29 human colon cancer cells[46].

Despite their biological importance, determining the targets of miRNAs is a major challenge. The problem stems from the discovery that functional mRNA regulation requires interaction with just 6 nucleotides of miRNA seed sequence[47]. In this study, we provide multiple pieces of evidence to identify miR-365 as a negative regulator of Rasd1. First, a miR-365 response element was predicted in the Rasd1 3'UTR with a seed sequence match by four common miRNA target prediction programs (miRDB, miRanda, MicroCosm and TargetScan). Second, miR-365 and its seed region are strictly conserved across species. The putative binding site of miR-365 to the Rasd1 3'UTR is broadly conserved among vertebrates. Third, the activity of a luciferase reporter in which the Rasd1 3'UTR was placed downstream of the reporter gene was repressed by miR-365 overexpression, while a luciferase reporter with miRNA-recognition element (MRE) mutation in the Rasd1 3'UTR was no longer repressed by miR-365. Finally, although the lack of appropriate antibodies for Rasd1 precluded further work to explore miR-365 effects on the endogenous protein, expression of miR-365 in MEFs was found to inversely correlate with Rasd1 mRNA levels. Therefore, our results provide experimental support for the prediction that Rasd1 is a direct target of miR-365.

Rasd1, also called dexas1 or AGS1, is a member of the Ras superfamily of small GTPases [48]. It was first discovered as a dexamethasone-inducible cDNA in AtT-20 mouse corticotroph cells [49] and subsequently as a receptor-independent activator of heterotrimeric G-protein signaling [50]. Koga et al found that RASD1 gene located in the region associated with a high incidence of loss of heterozygosity and deletions in cancers[51]. Moreover, Rasd1 can suppress cell growth and promote cell apoptosis in several different types of cells [52, 53]. Rasd1 interferes with the proliferation of MCF-7 cells through the downregulation of estrogen receptor alpha[54]. Recent studies also found that formononetin and calycosin can trigger cell apoptosis by up-regulating RASD1 in human cancer cells [55, 56]. In our study, we found that miR-365 suppressed the expression of Rasd1 through conserved seed matches within the 3'UTR of Rasd1, suggesting a model that miR-365 may modulate cell growth phenotypes including senescence in part by inhibiting expression of Rasd1.

In summary, massively parallel sequencing technology allowed us to accurately detect miRNAs expressed in MEFs. Considering the increasing use of MEFs for functional studies, our data provides a resource to design gene expression studies and to study gene regulatory networks mediated by miRNAs in these cells. Furthermore, our results from MEFs established from *Zmpste24*^{-/-} mice may generate an opportunity to identify miRNAs and pathways that are altered in HGPS and other diseases of aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MEFs	mouse embryonic fibroblasts
HGPS	Hutchinson-Gilford progeria syndrome
SA-β-gal	senescence-associated β -galactosidase
MRE	miRNA-recognition element

Highlights

- A comprehensive miRNA transcriptome of MEFs from *Zmpste24*^{-/-} and control mice
- Identification of miR-365 as a down-regulated miRNA in *Zmpste24*^{-/-} MEFs
- Characterization of miR-365 as a modulator of cellular growth in part by targeting *Rasd1*

Fig.1. Distribution of top 10 miRNAs expressed in wild type and *Zmpste24*^{-/-} MEFs
Top 10 miRNAs constitute nearly 80% of all miRNAs expressed in both MEFs established from wild type (WT) and *Zmpste24*^{-/-} progeroid mice.

Fig. 2. Expression levels of miRNAs in wild type and *Zmpste24*^{-/-} MEFs

The results were normalized against the expression level of U6 snRNA and the values were presented as mean fold change $\pm$ SEM ($n = 3$). Statistical analysis was performed by the student's *t*-test; * $P < 0.05$.

Fig. 3. MiR-365 modulates senescence in MEFs

(A) miR-365 expression levels after inhibition of miR-365 in wild type MEFs (left panel) and overexpression of miR-365 in *Zmpste24*^{-/-} MEFs (right panel). MiR-365 inhibitor (50nM) or miR-365 mimics (10nM) were transfected into WT MEFs and *Zmpste24*^{-/-} MEFs, respectively. MiR-365 expression levels were measured by qRT-PCR at 2 days after transfection. (B) Photomicrographs of SA-β-gal staining. WT and *Zmpste24*^{-/-} MEFs were treated with NC inhibitor/miR-365 inhibitor and NC mimics/miR-365 mimics, respectively. The cells were fixed and stained for senescence-associated β-gal at 6 days after transfection. (C) Percentages of SA-β-gal positive cells. ***P* < 0.01, ****P* < 0.001 by Student's *t* test. Values represent the mean ± SEM of three independent experiments. NC indicates negative control oligonucleotide.

Fig. 4. miR-365 mimics increase proliferation of *Zmpste24*^{-/-} MEFs

(A) Representative images of cells stained with hoechst (blue fluorescence) and EdU, as a measurement of DNA synthesis (red fluorescence). (B) Quantification of proliferation (EdU positive) as percentage of hoechst positive cells visualized in (A). Statistical analysis was performed by the student's *t*-test; Values represent the mean $\pm$ SEM of three independent experiments.

Fig. 5. Rasd1 is a target of miR-365

(A) Alignment of the Rasd1 3' UTR showed that the MRE (box) was conserved among different species. (B) Schematic representation of the hybridization between miR-365 and Rasd1 3' UTR. Green and red letters indicate miR-365 and Rasd1 mRNA sequences, respectively. (C) Schematic representation of mutant reporters of Rasd1 3'UTR. (D) Intact or mutant pGL3 cm-Rasd1 3'UTR and pRL-TK were co-transfected with the NC or miR-365 mimics into C2C12 cells. Luciferase activities were measured at 48h post transfection. (E) Luciferase reporter assays for the wild-type 3'-UTR of Pten and Sgk1. Statistical analysis was performed by the student's *t*-test; Data represent means $\pm$ SD (n=3); ***P* < 0.01 as compared with the transfection with the NC mimics.

Fig. 6. The effect of miR-365 on the endogenous expression of Rasd1

(A) Rasd1 levels of the wild type and *Zmpste24*^{-/-} MEFs (n=3). Rasd1 level was determined by qRT-PCR. Data were normalized to the β -actin mRNA. (B) The changes of Rasd1 after transfected with miR-365 inhibitor or miR-365 mimics. Fold changes in mRNA expression of Rasd1 were determined by qRT-PCR and normalized against the expression level of β -actin, and the values are presented as mean fold change $\pm$ SEM (n=3). Difference in the expression of the Rasd1 gene in the miR-365 inhibitor or miR-365 mimics group was compared with the transfection with their control. ** $P < 0.01$ by the student's *t*-test.

Table 1
Differentially expressed miRNAs in wild type vs. *Zmpste24*^{-/-} MEFs (Fold Change ≥ 2, p-value < 0.05).

miRNA name	Upregulated In	Wild type	<i>Zmpste24</i> ^{-/-}	pValue	Bonferroni Correction	Fold Change
miR-671-5p	<i>Zmpste24</i> ^{-/-}	34	148	3.32E-23	1.02E-20	4.42
miR-142-5p	<i>Zmpste25</i> ^{-/-}	15	33	0.001293	3.96E-01	2.15
miR-466f-5p	wild type	95	24	1.02E-08	3.12E-06	-4.01
miR-139-5p	wild type	43	13	0.000705	2.16E-01	-3.30
miR-200b	wild type	31	11	0.012792	1.00E+00	-2.88
miR-342-5p	wild type	111	42	6.45E-06	1.97E-03	-2.65
miR-582-5p	wild type	211	87	1.25E-08	3.82E-06	-2.42
miR-451	wild type	142	67	9.30E-05	2.85E-02	-2.14
miR-365	wild type	4080	2025	9.05E-85	2.77E-82	-2.02
miR-374	wild type	2872	1443	8.51E-58	2.60E-55	-2.00

Table 2

Representative GO categories enriched in targets of differentially expressed miRNAs.

Go Category	Go Annotation	p-Value
cell differentiation	GO:0030154	1.62E-09
gene expression	GO:0010467	8.55E-09
cellular metabolic process	GO:0044237	1.20E-06
cell-cell signaling	GO:0007267	5.00E-05
cell proliferation	GO:0008283	2.39E-05
cell cycle	GO:0007049	7.59E-06