AGE
DOI 10.1007/s11357-013-9555-6

MicroRNA-339 and microRNA-556 regulate Klotho expression in vitro

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Received: 6 December 2012 / Accepted: 18 June 2013

Abstract Klotho is an anti-aging protein with direct effects on life-span in mice. Klotho functions to regulate pathways classically associated with longevity including insulin/IGF1 and Wnt signaling. Decreased Klotho protein expression is observed throughout the body during the normal aging process. While increased methylation of the Klotho promoter is reported, other epigenetic mechanisms could contribute to age-related downregulation of Klotho expression, including microRNA-mediated regulation. Following in silico identification of potential microRNA binding sites within the Klotho 3′ untranslated region, reporter assays reveal regulation by microRNA-339, microRNA-556, and, to a lesser extent, microRNA-10 and microRNA-199. MicroRNA-339 and microRNA-556 were further found to directly decrease Klotho protein expression indicating that, if upregulated in aging tissue, these microRNA could play a role in age-related downregulation of Klotho messenger RNA. These microRNAs are differentially regulated in cancer cells compared to normal cells and may imply a role for microRNA-mediated regulation of Klotho in cancer.

Keywords Age downregulation · Luciferase · Untranslated region · Epigenetic

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Introduction

Klotho (KL) is an anti-aging protein that, in mice, increases life span when overexpressed and dramatically decreases life span when absent (Kuro-o et al. 1997; Kurosu et al. 2005). In addition to changes in life span, the absence of KL in mice results in an array of disorders normally associated with human aging including arteriosclerosis, osteoporosis, emphysema, and cognitive impairment (Kuro-o 1997; Nagai et al. 2003). KL is a type I transmembrane protein (Kuro-o 1997) that is shed from the cell surface to function as a humoral factor (Chen et al. 2007; Kuro-o 2009). As a result, disorders observed in the absence of KL affect nearly every organ system, even those that do not produce KL endogenously. In addition to observations in mice, rare human cases of profound KL protein alterations result in life-threatening disorders including hypophosphatemic rickets (Brownstein et al. 2008) and tumoral calcinosis (Ichikawa et al. 2007) with decreased and increased KL protein, respectively. Additionally, more common human polymorphisms affect risk of disease development (Kim et al. 2008; Kim et al. 2006; Ko et al. 2012).

Published online: 02 July 2013

Our lab has reported an age-dependent decrease in KL messenger RNA (mRNA) and protein within white matter of aged rhesus monkey and rodents (Duce et al. 2008; King et al. 2011). Age-related decreases in KL have likewise been reported in aged rodent heart and liver (Nabeshima 2002; Shih and Yen 2007). The KL promoter and first exon lay within a CpG island indicating a possible mechanism of pre-transcriptional epigenetic silencing as a reasonable candidate to explain age-related downregulation (King 2011). This possibility is strengthened by recent work indicating that promoter methylation may, in part, explain why expression of KL is restricted to a limited number of organs (Azuma et al. 2012). Beyond normal processes, regulation of KL through methylation of its promoter is reported in multiple cancers (Camilli et al. 2010; Chen et al. 2010; Lee et al. 2010; Wolf et al. 2008).

Another epigenetic regulatory possibility to explain age-related downregulation lies on the other end of the gene in the 3′ untranslated region (3′ UTR). The human KL gene is composed of five exons and four introns (Matsumura et al. 1998). The KL coding sequence results in an mRNA of 5,006 bp of which nearly 2,000 bp are 3′ UTR (NM_004795.3). MicroRNA (miR) are small, endogenous, noncoding, single-stranded RNA molecules that interact with binding sites within 3′ UTRs to post-transcriptionally regulate gene expression (Stefani and Slack 2008). MiRs are generated from stem-loop structures that are processed first in the nucleus and then the cytoplasm. Processing leads to strand selection and incorporation of a single-stranded miR into the RNA-induced silencing complex. Partial complementarity of miR with mRNA transcripts inhibits mRNA translation by targeting mRNA for degradation. Although hundreds of miRs have been identified through expression profile analysis, functional characterization of individual miRs is limited. Expression patterns of miRs are unique from cell type to cell type and are found to change in disease states, across development, and into normal aging (Boehm and Slack 2006). Age downregulation of KL may be a result of miR expression changes with age. Although the absence of KL in mice alters miR expression (Gui et al. 2012; Takahashi et al. 2012), to date no information is available as to whether miRs regulate KL expression. Here, we report in vitro evidence of regulation of KL mRNA by miR-339 and miR-556. As these miRs are found in cancers, it is likely that altered miR expression with age and/or disease could affect KL expression and thus alter its functions.

Methods

Cell culture and plasmid constructs

HEK293T cells were cultured in DMEM containing 10 % FBS and 1 % penicillin/streptomycin. Cells were maintained under standard growth conditions. All media and media components were obtained from Mediatech. The human KL 3′ UTR luciferase reporter construct in pSSG and RO1 random 3′ UTR control vector were generated by Switchgear Genomics. The SV40-driven Renilla luciferase plasmid was obtained from Promega. To generate binding site mutants, site-directed mutagenesis was performed per manufacturer's instructions (Stratagene). Primers to generate binding site mutations were as follows:

miR-10a	5′ GCCAACCTCACTRGACACACCG TCATAGTGTATAATAAT 3′
miR-339	5′ GTCTTGCTGATTTTCAGACTCGG AAGTCTCTCTAT 3′
miR-556	5′ CATGAAAGATAAGCTTTTGGTTT TATTTCATTTTAAAGTGGAC 3′

Primers were obtained from Integrated DNA Technologies (IDT). The full-length KL construct was generated by restriction digestion of the KL cDNA in pcDNA3.1 V5His (Life Technologies) and the KL 3′ UTR reporter construct with EcoRI and XhoI (New England Biolabs, NEB). Following CIP treatment of the KL cDNA plasmid, sticky ends were ligated using Quik ligase (NEB). Restriction digestion and sequencing confirmed successful integration of the 3′ UTR using primers in the coding region of the fifth exon in Klotho and the BGH reverse primer site located in the plasmid.

In silico analysis

Using online prediction tools miRanda (August 2010 release) (John et al. 2004), TargetScan (release 6.2) (Lewis et al. 2005), PicTar (Krek et al. 2005), and DIANA-microT CDS (version 5) (Kiriakidou et al. 2004), 4–251 possible miR binding sites were predicted within the KL 3′ UTR. MiRs with the highest probability of binding in at least two independent

algorithms were prioritized for further analysis. Seed regions and corresponding sequences within the KL 3′ UTR were identified using miRBase and Ensembl (Griffiths-Jones 2004).

KL 3′ UTR reporter assay

Based on the presence of a potential binding site, miR precursors to miR-10a, miR-199, miR-203, miR-339, miR-556, miR-587, and a scrambled miR control were obtained (Life Technologies). Of miR precursors, 30 and 60 nM were transfected with the KL 3′ UTR luciferase reporter to determine a minimal concentration of miR to observe an effect. Consistent with manufacturer's instructions, a concentration of 30 nM miR precursor was sufficient. HEK293T cells (ATCC) were plated in 96 well plates and transfected using Attractene (Qiagen) 24 h later. Cells were triple transfected with the KL 3′ UTR firefly luciferase reporter construct (400 ng), Renilla luciferase control (100 ng), and 30 nM of miR precursor. Control conditions utilized the RO1 random 3′ UTR firefly luciferase control vector in place of the KL 3′ UTR. All conditions were run in triplicate. Twenty-four hours after transfection, cells were lysed using passive lysis buffer and both luciferase reporter constructs quantified using Dual Luciferase (Promega) on an Spectramax M3 plate reader (Molecular Devices). To control for transfection efficiency, the firefly luciferase value was normalized to the Renilla luciferase value for each well. Samples were then normalized to their relevant control in which the scrambled miR was transfected. At least three independent assays were performed. To assess the effect of binding site mutation, similar assays were performed utilizing mutated and non-mutated luciferase reporter constructs transfected with Renilla luciferase in the presence of either the miR of interest or the scrambled miR.

miR detection in HEK293T cells

To assess endogenous levels of miR-339 and miR-556, microRNAs were isolated from HEK293T cells using the miRNeasy kit per manufacturer's instructions (Qiagen). MiR-339 and miR-556 levels were assessed using miScript and miR-specific primer sets miR-556 (Ms00032004, Qiagen), miR-339 (Ms0003997, Qiagen), and miR-15A (miScript primer assay, Qiagen) by SYBR green qPCR, per manufacturer's instructions (Qiagen). Results are reported as average Ct value from two independent experiments.

miR effect on KL mRNA

To assess miR function on KL mRNA expression, the full-length KL plasmid expressing the KL cDNA with the the 3′ UTR intact following the natural stop codon as detailed above was co-transfected into HEK293T cells with 30 nM of a miR precursor or scrambled control. Twenty-four hours later, mRNA was isolated per manufacturer's instructions using the RNeasy kit (Qiagen). mRNA quality was assessed by 260/280 nm ratio. Total mRNA was converted to cDNA as per manufacturer's conditions using iScript RT (Biorad) and cDNA was used for qPCR reaction using SsoFast Probes Supermix with ROX (Biorad). Primers/probes were obtained from IDT. Primer/probes utilized were as follows: FAM-labeled human Klotho, primer 1: 5′-GAGTGGTATCTACTTGAATGTAGT-3′, primer 2: 5′-GTCTTCAGCC TTG TTCTACC-3′ and probe 5′-56-FAM/CCCAAGCAA/ZEN/AGTCACAGGGAA ATGT C/3IABkF-3′ or HEX-labeled human 18 s subunit, primer 1: 5′-GGACATCTAAGGGCATCACAG-3′, primer 2: 5′-GAGACTCTGGCATG CTAACTAG-3′ and probe 5′-5HEX/TGCTCAATC/ZEN/TCGGGT GGCTGAA/3IABkFQ-3′. Assays were conducted using StepOne qPCR system (Applied Biosystems). Cycle conditions were 95 °C for 2 min, 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. Ct values were obtained for each condition and ΔCt value was calculated as the difference between Ct values of 18s and human Klotho. ΔΔCt was used to calculate fold difference relative to scrambled control.

miR effect on KL protein

To assess miR function on KL protein expression, the full-length KL plasmid expressing the KL cDNA with the 3′ UTR intact following the natural stop codon as detailed above was co-transfected into HEK293T cells with 30 nM of a miR precursor or scrambled control. Twenty-four hours later, cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1 % Triton X-100, 0.5 % sodium deoxycholate, and 1 % sodium dodecyl sulfate). Following BCA assay (Pierce) to quantify total protein, 30 μg of total cell lysate was loaded onto a 10 % polyacrylamide gel. Proteins were

transferred to nitrocellulose (Millipore) and blocked with 5 % nonfat dry milk in Tris-buffered saline containing 0.05 % Tween 20. Transfection of the plasmid results in the anticipated 130kDa KL protein band detected by western blotting using the Klotho-specific KM2076 (Sceti K.K.). KL expression was normalized to β-tubulin expression (Developmental Studies Hybridoma Bank) for each lane sample. To allow for comparison across multiple experiments, the KL full-length with scrambled control intensity was considered 100 %. Band intensity was quantified using ImageJ version 1.46. Statistical significance by one-way ANOVA was detected comparing all groups ($p<0.0001$). Post hoc analysis to determine whether groups were different from scrambled control found a significant decrease compared to the negative control, pcDNA ($p<0.0001$) and miR-339 and miR-556 ($p<0.005$).

Statistics

Statistical significance was calculated using Graphpad Prism software version 5.0. *t* test or ANOVA with Dunnett's test for post hoc analysis was used where indicated, to determine if minimum threshold of at least $p<0.05$ had been achieved between groups.

Results

The human KL gene is composed of four introns and five exons (Matsumura et al. 1998). The promoter and much of exon 1 are within a CpG island. The fifth exon is the largest spanning 2,296 bp; 1,958 bp compose the 3′ UTR, falling between the stop codon and polyadenylation signal (Fig. 1). We utilized multiple online miR binding site prediction algorithms to determine whether binding sites for known miRs were present in the KL 3′ UTR (John et al. 2004; Kiriakidou 2004; Krek et al. 2005; Lewis et al. 2005). MiR binding site prediction tools miRanda and TargetScan base predictions on binding site complementarity while DIANA microT and PicTar utilize thermodynamics to predict binding (Maziere and Enright 2007). We prioritized six miRs (miR-10, miR-199, miR-203, miR-339, miR-556, miR-587) for further evaluation based on score and presence on multiple prediction lists. For these six miRs, nine sites consistent with miR binding are present in the KL 3′ UTR (Table 1). A single binding site is predicted for miR-10, miR-339, and miR-556 (Fig. 1), while two possible sites are identified for miR-199, miR-203, and miR-587 (Fig. 1).

In our hands, KL mRNA and protein expression in immortalized human cell lines, including HEK293T, is low to undetectable. As an interaction with miR is anticipated to decrease expression, attempting to use

CCCACATACTGGATGGTATCAATCTTTGCGGATACTTTGCTTATTCGTTTAACGACCGCACAGCTCCGAGGTTTGGCCTCTATCGTTATGCTGC
AGATCAGTTTGAGCCCAAGGCATCCATGAAACATTACAGGAAAATTATTGACAGCAATGGTTTCCCGGGCCCAGAAACTCTGGAAAGATTTTGT
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*CTCTCTCCCTTATATTTTACTACTCGAAGAAAGGCAGAAGAAGTTACAAA**TAG**TTCTGAACATTTTTCTATTCATTCATTTTGAAATAATT*
ATGCAGACACATCAGCTGTTAACCATTTGCACCTCTAAGTGTTGTGAAACTGTAAATTTCATACATTTGACTTCTAGAAAA
CATTTTTGTGGCTTATGACAGAGGTTTTGAAATGGGCATAGGTGATCGTAAAATATTGAATAATGCGAATAGTGCCTGAAT
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GCCTAGTGGCTTCCCCTCTGTCAAATCTAGTTTCCTATGGAAAAGAAGATGGCAGATACAGGAGAGACGACAGAGGG
TCCTAGGCTGGAATGTTCCTTTCGAAAGCAATGCTTCTATCAAATACTAGTATTAATTTATGTATCTGGTTAATGACATACT
TGGAGAGCAAATTATGGAAATGTGTATTTTATATGATTTTTGAGGTCCTGTCTAAACCCTGTGTCCCTGAGGGATCTGT
CTCACTGGCATCTTGTTGAGGGCCTTGCACATAGGAAACTTTTGATAAGTATCTGCGGAAAAACAAACATGAATCCTGT
GATATTGGGCTCTTCAGGAAGCATAAAGCAATTGTGAAATACAGTATACCGCAGTGGCTCTAGGTGGAGGAAAGGAGG
AAAAAGTGCTTATTATGTGCAACATTATGATTAATCTGATTATACACCATTTTTGAGCAGATCTTGGAATGAATGACATGAC
CTTTCCCTAGAGAATAAGGATGAAATAATCACTCATTCTATGAACAGTGACACTACTTTCTATTCTTTAGCTGTACTGTAAT
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ATCTCTGAGGTCTATTTTATGTTTTTGCTGCTACTTCTGTGGAAGTAGCTTTGAACTAGTTTTACTTTGAACTTTCACGCT
GAAACATGCTAGTGATATCTAGAAAGGGCTAATTAGGTCTCATCCTTTAATGCCCCTTAAATAAGTCTTGCTGATTTTCAG
ACAGGGAAGTCTCTCTATTACACTGGAGCTGTTTTATAGATAAGTCAATATTGTATCAGGCAAGATAAACCAATGTCAT
AACAGGCATTGCCAACCTCACTGACACAGGGTCATAGTGTATAATAATATACTGTACTATATAATATATCATCTTTAGAGG
TATGATTTTTTCATGAAAGATAAGCTTTTGGTAATATTCATTTTAAAGTGGACTTATTAAAATTGGATGCTAGAGAATCAA
GTTTATTTTATGTATATATTTTTCTGATTATAAGAGTAATATATGTTCATTGTAAAAATTTTTAAAACACAGAAACTATATGCAA
AGAAAAAATAAAAATTATCTATAATCTCAGAACCCAGAAATAGCCACTATTAACATTTCCTACGTATTTTATTTTACATAGAT
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ATGAATGTTGTTTAAAAATAATTTTGTTGCTACATTTACTTTAATTTCCTTGACTGTAAAGAGAAGTAATTTTGCTCCTTGAT
AAAGTATTATATTAATAATAAATCTGCCTGCAACTTTTTGCCTTCTTTCATAATC

Fig. 1 Sequence of human KL exon 5. The fifth and final exon of KL is comprised of bp 2710–5506 (NM_004795.3). The coding region is *italicized* and the stop codon is in *bold and underlined*. The 1,956 bp from the stop codon to polyA sequence comprise the 3′ UTR. Online miR binding site prediction algorithms were used to identify potential binding sites present in the KL 3′ UTR (*underlined*, *larger font*)

Table 1 miR binding sites in KL 3′ UTR

miRNA	Seed region sequence	Sequence in KL 3′ UTR
miR-10-5p	5′-ACCCUGU-3′	5′- GACACAGGGTCAT- 3′
miR-199-5p	5′-CCAGUGU-3′	5′-ATAACACTGGATTT-3′
		5′- ATTACACTGGAGCT- 3′
miR-203-5p	5′-GAAAUG-3′	5′- AACCATTTCTCA- 3′
		5′-TAACATTTCCTA- 3′
miR-339-5p	5′-CCCUGUC-3′	5′- TCAGACAGGGAAG- 3′
miR-556-3p	5′-UAUUACC-3′	5′- TTTGGTAATATTC- 3′
miR-587-5p	5′-UUCCAUA-3′	5′-TCCTATGGAAAAG-3′
		5′- AATTATGGAAATG-3′

endogenous expression of KL in cell lines was not reasonable. To determine whether the KL 3′ UTR can be regulated by miR, we constructed a luciferase reporter plasmid in which the KL 3′ UTR was cloned after the firefly luciferase stop codon. Firefly luciferase activity, as detected by luminescent light output, thus serves as a surrogate for effects on KL. If miR are able to interact with the mRNA containing the KL 3′ UTR, light output from the luciferase protein would be altered. We triple transfected the luciferase reporter, a constitutively expressing Renilla luciferase plasmid, and double-stranded miR precursors (Fig. 2a, white bars) and measured luciferase activity. An irrelevant control UTR luciferase plasmid was likewise transfected with Renilla luciferase and miR precursors (Fig. 2a, gray bars). Well to well transfection efficiency was controlled by normalizing firefly luciferase to Renilla luciferase. Average luciferase values were normalized to luciferase transfected with scrambled miR control for each experiment. Upon transfection with the KL 3′ UTR, miR-339 and miR-556 specifically decreased luciferase protein production as measured by luminescent output (Fig. 2a). Luminescence detected from KL 3′ UTR transfected wells was also decreased upon transfection with miR-10 and miR-199; however, similar decreases were observed using the control UTR suggesting a nonspecific effect (Fig. 2a). To verify that the decrease in luciferase was due to a specific interaction of the miR with the predicted binding site in the KL 3′ UTR, we mutated the binding site by changing 2 bp in the seed region. Mutated and wild-type plasmids were transfected with and without miR precursors and light output measured as above. As anticipated, mutation of the binding site for miR-339 or miR-556 restored wild-type levels of luciferase expression (Fig. 2b, stripped bars). Suspecting a lack of specificity responsible for the results with miR-10 and miR-199, we mutated the mirR-10 binding site, as a representative, and saw an increase in luciferase expression indicating a markedly smaller but likely specific effect on

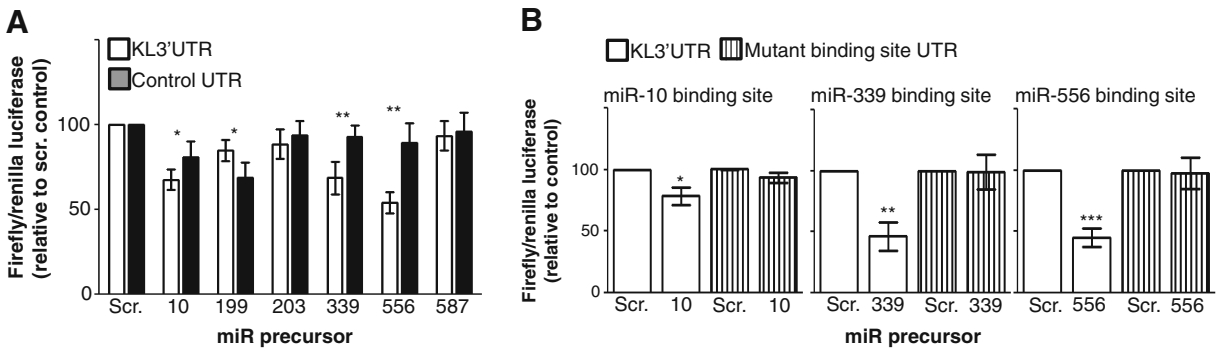

Fig. 2 MiR-339 and miR-556 decrease KL 3′ UTR-mediated luciferase expression. **a** To assess whether miRs predicted to bind the KL 3′ UTR would posttranscriptionally regulate KL, we transfected HEK293T cells with KL 3′ UTR reporter constructs (*white bars*) and either a miR precursor or scrambled (*Scr.*) control. The R01 plasmid containing a scrambled 3′ UTR with no miR binding sites (*gray*) was used as a control. Samples were run in triplicate for each assay and each assay was performed independently, three times; *bar* represents the mean ±SEM. Statistical significance was determined by *t* test comparing the KL 3′ UTR vs. the control UTR for each miR precursor; *$p<0.05$, **$p<0.01$. **b** To determine whether binding of miR to the consensus binding site was responsible for the change in luminescence observed, each binding site was mutated for the miR-10, miR-339, and miR-556. The non-mutated reporter plasmid was transfected with and without the relevant miR (*white bars*). Concomitantly, the mutated reporter plasmid was transfected with and without the relevant miR (*stripped bars*). Four independent experiments were conducted; *bar* represents the mean±SEM. Statistical significance was determined by *t* test between scr and miR precursor for each UTR or mutated UTR; *$p<0.01$, **$p<0.001$, ***$p<0.0001$

reporter light output (Fig. 2b). We used qPCR to determine whether either miR-339 or miR-556 is expressed in HEK293T cells. Positive control miR-15A, assumed to be detectible in all tissues, crossed fluorescent threshold at cycle 27 (n=2; ±1.41 SD). Meanwhile, miR-339 was found more abundant having crossed threshold at cycle 25 (n=2; ±1.41 SD) and miR-556 was less abundant not detected until cycle 31 (n=2; ±1.41 SD).

Although informative and important for validation, use of the luciferase reporter assay may not directly translate into effects on KL mRNA and protein. As stated, we are unable to detect either endogenous KL mRNA or protein in HEK293T cells. To allow us to evaluate an effect of mirR-339 and miR-556 on KL expression, we modified our plasmid that expresses the full-length KL coding sequence. To do so, we cloned the KL 3′ UTR from the luciferase reporter to be contiguous with the natural KL stop codon. The 2-kbp addition was verified by both restriction digestion and sequencing. The full-length KL plasmid (KLFL) was transfected into HEK293T cells with either a miR or scrambled precursor. Twenty-four hours after transient transfection, cell lysates were processed for qPCR to evaluate mRNA levels or western blot to assess effect on translation and protein. KL and 18S ribosomal subunit mRNA levels were detected in a multiplex qPCR reaction using specific primer/probes for each mRNA in each sample. Relative quantification normalizing first the 18S Ct value and then the scrambled control and was used to determine whether differences were observed upon transfection with the various miR. Across four independent transient transfections, no significant difference was observed (Fig. 3a). As our experiments utilized transient transfection and protein levels are ultimately the most important outcome measure of regulation, in parallel transfections, KL protein levels were assessed by western blot. As anticipated, HEK293T cells did not make detectible amounts of KL protein but transfection of the KLFL resulted in a 130-kDa band consistent with proper translation removing the 3′ UTR present in the mRNA (or this case the plasmid; Fig. 3b). Presence of miR-339 or miR-556 but not miR-10 or miR-199 specifically decreased KL protein expression (Fig. 3b, c).

Discussion

In mice, the absence of KL results in altered miR expression. In the aorta, the absence of KL induces upregulation of miRs that regulate calcium ion transporters promoting an environment in which calcification can occur (Gui et al. 2012). Likewise, expression profiling across multiple organs in KL knockout mice indicate an increase in a number of miRs, most prominently of the miR-29 family (Takahashi et al. 2012). MiR-29 family members are also increased in 18-month-old outbred ICR mice indicating this family of miRs may be involved in aging mechanisms (Takahashi 2012). While this previous work indicates that KL is important in regulating miR expression, a direct effect of miR on KL has not been described prior to the present study. We show that KL is regulated by miR-339, miR-556, and to a much lesser extent, by miR-10 and miR-199 (Fig. 2). Interaction of miR-339 and miR-556 with the 3′ UTR of KL mRNA decreases protein expression in vitro (Fig. 3). The six miRs selected for investigation in this study were predicted across multiple online prediction tools that used either complementarity of binding site or thermodynamics (Maziere 2007). All prediction tools have a false-positive rate somewhere between 20 and 40 % (Maziere 2007), consistent with what we observed based on reporter assay results. In our hands, neither method of prediction appears to be more consistent in picking the miRs that actually impacted KL expression. As well, selecting based on miR predicted in multiple algorithms did not decrease the false-positive rate.

In humans, miR-339 is derived from the intron of a putative protein on chromosome 7 (Kozomara and Griffiths-Jones 2011). After identification in rat neurons (Kim et al. 2004), miR-339 orthologs were identified in mouse and human tissue (Weber 2005). Expression profiling reports detection of miR-339 in adult hippocampus (Landgraf et al. 2007), cervix (Witten et al. 2010), placenta (Cummins et al. 2006), and in numerous cancers (Kasashima 2004; Landgraf 2007; Stark et al. 2010). MiR-339 is reported to regulate ICAM-1 (Ueda et al. 2009), BCL6 (Wu et al. 2010), and μ-opioid receptor (Wu 2012). Relevant to our interests in KL as an age-related protein, miR-339 is reported to be among only 16 miR upregulated in long-lived humans, showing a 2.37-fold increase in this cohort (ElSharawy et al. 2012).

KL has also been implicated as a tumor-suppressor protein in numerous cancers (Camilli 2010; Chang et al. 2012; Chen 2010; Lee 2010; Wang 2011; Wolf 2008) where decreased expression promotes tumorigenesis.

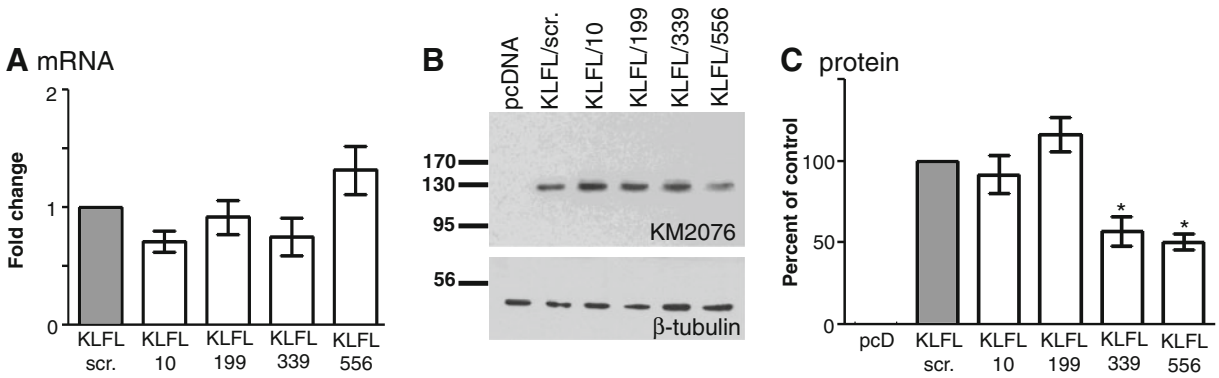

Fig. 3 MiR-339 and miR-556 decrease KL protein. We generated a full-length KL plasmid (*KLFL*) containing the KL coding sequence with its 3′ UTR. Full-length KL was transfected into HEK293T cells with miR precursors or a scrambled miR control (*scr*). **a** Multiplex qPCR was conducted measuring KL and 18S ribosomal subunit quantity in transiently transfected cells. Fold change was calculated by ddCt. No statistical difference was detected in mRNA. Four independent experiments were conducted; *bar* represents the mean±SEM. Statistical significance was determined by one-way ANOVA, statistical significance failed to reach $p<0.05$ and post hoc test did not detect differences relative to scrambled control. **b** Representative western blot showing KL and β-tubulin protein expression from cells transiently transfected with miR. **c** Band intensity was quantified across multiple independent experiments using ImageJ. The band intensity for KL was normalized to the β-tubulin intensity for each sample. Four independent experiments were conducted; *bar* represents the mean±SEM. Statistical analysis by ANOVA with Dunnett's multiple comparison test indicates a difference between groups; *$p<0.002$

The role of KL in glioma is unknown; however, miR-339 has been investigated in glioma cell lines and tumor samples. MiR-339 is upregulated in glioma cell lines and tissues and its upregulation causes the downregulation of ICAM-1 (Ueda 2009). When ICAM-1 levels are decreased, critical interaction of ICAM-1 with LFA-1 on tumor-specific cytotoxic T cells cannot occur, allowing tumor cell evasion (Ueda et al. 2009). Conversely, miR-339 is downregulated in breast cancer and breast cancer cell lines with least expression in the most aggressive and metastatic tumors (Wu 2010). Anti-sense knockdown of miR-339 in cell lines increased migration and invasion (Wu 2010). This increase in migration may be attributable to the decrease in miR-339 resulting in an increase in oncogene BCL-6 (Wu 2010). Intriguingly, KL is also downregulated in breast cancer tissues and in some of the same cell lines used to assess miR-339. Inconsistent with what we would have predicted for a direct effect of miR-339 on KL, MCF-7 cells were shown to have the highest levels of KL mRNA of the tumor lines tested (Wolf 2008) and have the highest levels of miR-339 (Wu 2010). The disparate findings between glioma and breast cancer cells indicate that miR expression is highly specific to cell type and that other factors may be involved in controlling KL expression particularly in the cancer cell environment.

Although work to characterize miR-339 is reported, very little is known about miR-556. MiR-556 was first described in an expression profiling experiment with human colorectal cancer cells (Cummins 2006) and subsequently in other cancers including cervical and melanoma (Landgraf 2007; Stark 2010; Witten et al. 2010), although its expression levels in all studies is very low. It is derived from intron 5 of the nitric oxide synthase 1 adaptor protein. MiR-556 is not reported in noncancerous cells. As reported on the miR registry, miRBase, although it has orthologs in nonhuman primates, it has not been discovered in other mammals (Kozomara and Griffiths-Jones 2011). KL is downregulated in cancers which may be consistent with the presence of miR-556 regulating KL expression in cancer cells; however, the low abundance of miR-556 would indicate that further studies are needed to determine whether it plays such a role in vivo.

In addition to the μ-opioid receptor, BCL-6, and ICAM-1, our work would suggest that KL is the fourth known protein to be regulated by miR-339 and the first protein to be regulated by miR-556. It is possible that these two, rare miRs could impact KL function in specific cell types or disease conditions; however, at this time, it is not possible to conclude that age-related downregulation of KL or its downregulation in cancers is simply attributable to an increase in specific miRs alone. This is not unexpected as both aging and

malignant transformation are complex processes. As well, miRs are promiscuous, able to bind to and regulate any number of proteins containing binding sites adding to the regulatory complexity. With KL promoter methylation described in cancer as well as normal tissues, a complex process of many different factors likely function together to regulate KL expression as one of many critical proteins in a complex network. This work would indicate that miR interaction with the KL 3′ UTR is possible, warranting further investigation in aging and cancer paradigms as well as with other candidate miRs.

Acknowledgments This work was funded by National Institutes of Health/National Institute on Aging R00 AG034989 (GDK).

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