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MicroRNA regulation in Ames dwarf mouse liver may contribute to delayed aging

David J. Bates^{1,2}, Na Li², Ruqiang Liang², Harshini Sarojini², Jin An², Michal M. Masternak³, Andrzej Bartke³, and Eugenia Wang^{1,2,†}

¹Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine

²Gheens Center on Aging, University of Louisville School of Medicine

³Geriatrics Research, Department of Internal Medicine and Department of Physiology, Southern Illinois University, School of Medicine, Springfield, IL

SUMMARY

The Ames dwarf mouse is well known for its remarkable propensity to delay the onset of aging. Although significant advances have been made demonstrating that this aging phenotype results primarily from an endocrine imbalance, the posttranscriptional regulation of gene expression and its impact on longevity remains to be explored. Towards this end, we present the first comprehensive study by microRNA microarray screening to identify dwarf-specific lead microRNAs, and investigate their roles as pivotal molecular regulators directing the long-lived phenotype. Mapping the signature microRNAs to the inversely expressed putative target genes, followed by *in situ* immunohistochemical staining and *in vitro* correlation assays, reveal that dwarf mice posttranscriptionally regulate key proteins of intermediate metabolism, most importantly the biosynthetic pathway involving ornithine decarboxylase and spermidine synthase. Functional assays using 3'UTR reporter constructs in co-transfection experiments confirm that microRNA-27a indeed suppresses the expression of both of these proteins, marking them as probable targets of this microRNA *in vivo*. Moreover, the putative repressed action of this microRNA on ornithine decarboxylase is identified in dwarf mouse liver as early as two months of age. Taken together, our results show that among the altered aspects of intermediate metabolism detected in the dwarf mouse liver — glutathione metabolism, the urea cycle, and polyamine biosynthesis — microRNA-27a is a key posttranscriptional control. Furthermore, compared to its normal siblings, the dwarf mouse exhibits a head start in regulating these pathways to control their normality, which may ultimately contribute to its extended healthspan and longevity.

Keywords

MicroRNA; Aging; dwarf mouse; intermediate metabolism

Introduction

Organismal senescence, the inevitable process of aging faced by every organism, may be characterized by a decreasing ability to handle stress (both exogenous and endogenous), and the increasing homeostatic imbalance, genetic malfunction, and risk of disease. Recent studies demonstrate that genetically-altered organisms with significantly longer life-spans

[†]Corresponding Author: Eugenia Wang, Ph.D., University of Louisville School of Medicine, 580 S. Preston St., Louisville, KY 40202, USA., Tel: 502-852-2554, Fax: 502-852-2555, Eugenia.Wang@Louisville.edu.

Supplementary Information will be linked to the online version of the paper.

also possess an enhanced ability to respond to environmental and cellular stresses, and minimize genetic alterations or error (Kenyon 2005). Understanding the mechanistic and molecular processes that enable these organisms to live longer may yield insight into ways to decrease the rate of aging and minimize its detrimental effects.

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs, usually between 18 to 25 nucleotides in length, involved in the regulation of cellular and developmental processes through posttranscriptional gene repression (Ambros 2004; Alvarez-Garcia 2005). MicroRNAs typically bind to partially complementary regions of the 3' untranslated region (UTR) of messenger RNAs (mRNAs), to induce either translation inhibition or signal for mRNA degradation. A more complete understanding of the role miRNAs play in the process of aging could lead to gene-based strategies to diagnose, suppress, or cure aging-related symptoms and disease.

The Ames dwarf mouse has been an attractive animal model in the search for genomic factors that control or affect the process of aging. Mice of this strain live up to 70% longer than wild-type counterparts, because of a deficiency in three pituitary hormones (growth hormone, prolactin, and thyrotropin), resulting from a point mutation affecting the *Prop1* gene (Schaible 1961; Anderson 1995). Recent findings demonstrate that some miRNAs either regulate (Poy *et al.* 2004) or are regulated by endocrine-related functions (Fiedler *et al.* 2008). The GH/IGF-1/insulin signaling pathway is reported to play a direct role in the rate of aging (Longo 2003; Tatar 2003). Different expression levels of growth hormone (GH) have been shown to directly affect insulin sensitivity (Dominici 2000; Dominici 2002), polyamine synthesis (Russell 1969; Russell 1970; Sogani 1972; Gritli-Linde 1997), and stress resistance (Murakami 2003; Brown-Borg 2005; Salmon 2005). Pertinent to this study, increases in GH correlate directly with increased levels of ornithine decarboxylase 1 (ODC1) and polyamines (Kostyo 1966; Russell 1969; Russell 1970; Gritli-Linde 1997). Imbalances in ornithine, hence polyamine metabolism, are linked to tumorigenesis and a suite of different cancers (Manteuffel-Cymborowska *et al.* 1995; Young *et al.* 2006).

Although many facets of the dwarf mouse warrant study (*e.g.*, tumor suppression, altered metabolism, increased memory, *etc.*), little is known about the posttranscriptional regulation of gene expression directing these phenotypic features. In this study, variations in protein and miRNA expression were investigated in dwarf mice of different ages, and contrasted against their wild-type counterparts *via* global proteomic profiling in conjunction with miRNA microarray analyses, followed by functional analysis of specific lead miRNA/target relationships. To study the effects of aging, we selected the liver because of its role in detoxification, hormone degradation, and its direct correlation to aging in mammals (Cao 2001; Schmucker 2005; Maes 2008).

Here we report the up-regulation of ten miRNAs in dwarf mouse liver, with microRNA-27a emerging as the leading significant miRNA species in this context. Parallel proteomic profiling reveals that genes targeted by these miRNAs exhibit reciprocal down-regulation, and are in protein families associated with intermediate metabolism—specifically glutathione metabolism, the urea cycle, and polyamine biosynthesis. *In situ* immunohistochemical staining and *in vitro* reporter and endogenous expression assays demonstrated an inversely correlated expression between miR-27a and two of its putative targets involved in polyamine biosynthesis— ODC1 and spermidine synthase (SRM). In particular, the reciprocal expression of miR-27a and ODC1 can be observed as early as 2-month old livers of dwarf mice, giving these mice a head start over their control wild-type brothers. Thus, the up-regulated miRNAs, led here by miR-27a, suggest that noncoding RNAs serve as posttranscriptional regulatory factors, functionally vital to longevity determination.

RESULTS

MicroRNA microarray analysis

Comparative expression analysis of screening by miRNA microarray (MMChips) for each of 15 mice, divided evenly into five categories (control 2-month-old [m.o.], dwarf 2-m.o., control 24-m.o., dwarf 24-m.o., dwarf 33-m.o.), reveals that the vast majority of miRNAs exhibiting variation in dwarf mice compared with controls are up-regulated (Figure 1. A–F and Supplementary Figure 3). Only two miRNAs, microRNA-29c (miR-29c) and miR-707, are downregulated in the aged dwarf compared with controls (Figure 1. E & F). Most miRNAs that exhibit variations in expression during aging typically increase in expression with age, and much more markedly in the dwarf. MicroRNA-27a exhibits the most drastic disparity between dwarf and control mice (Figure 2. A); this miRNA increases with age in the dwarf, whereas there is a slight decrease with age in controls. MicroRNAs postulated to target genes of the urea cycle (part of arginine metabolism) display increased expression with age (Figure 2. B and Supplementary Figure 3).

qRT-PCR Confirmation

MicroRNA microarray expression analysis was qualitatively confirmed by qRT-PCR of miRNAs mmu-miR-27a, -669b, -22, -96, and -501-3p. These five miRNAs were selected because they exhibit the strongest theoretical correlation with target genes that also exhibit inverse expression experimentally (Table 2 shaded in gray). MicroRNA-27a displays significantly increased expression in the dwarf mouse at 24 months of age over controls. A disparity exists between MMChip results and qPCR in miR-27a expression in the 24-month-old control compared to the 2-month-old control; the chip shows decreased expression in the older control, whereas qPCR displays increased expression (Figure 3. A). qPCR also shows miR-27a expression in 33-month-old dwarfs on par with 24-month-old controls, whereas the chip displays increased expression in the dwarf at that time point. In general, microarray screening is less sensitive, and often presents data according to analysis of data from the entire array; even with all the positive and negative controls, the results are the outcome of hundreds of loci on the arrays. The microarray is therefore less sensitive than qPCR, because with qPCR the data output quantifies a single miRNA. Mmu-miR-669b displays a similar pattern of expression, with levels peaking at 24 months in the dwarf and dropping sharply at 33 months of age, but still remaining much higher than the 24-month-old control (greater than fourfold) (Figure 3. B). Mmu-miR-22 displays expression similar to miR-27a, with maximum expression at 24 months of age, six times higher in the dwarf than in the same-aged control, followed by plummeting expression at 33 months, to levels nearly equal to 24-month-old controls (Figure 3. C). Mmu-miR-96 qualitatively corroborates the microarray data except for expression at 24 months of age in the dwarf mouse, which is on average 3.76-fold less than same age controls (Figure 3. D). Interestingly, 33-month-old dwarfs exhibit miR-96 expression nearly equal to that of controls at 24 months of age. Mmu-miR-501-3p displays increased expression in the dwarf at 2 and 33 months of age (1.75 and 3.74-fold compared to 24-month-old controls, respectively), whereas 24-month-old dwarfs exhibit miR-501-3p expression on par with same-aged controls (Figure 3. E).

Proteomic Results

Comparative proteomics of the Ames dwarf mouse and its wild-type counterpart reveals striking differences in expression of proteins involved in intermediate metabolism and detoxification. Enzymes involved in arginine and ornithine metabolism—specifically the urea cycle—show significant variation. Arginase 1 (ARG1), argininosuccinate synthase (ASS1), and carbamoyl-phosphate synthetase (CPS1) exhibit particularly varied expression patterns (Figure 4. A – C). In the young dwarf (2 months of age) these enzymes are considerably up-regulated compared to the young control. Expression of these proteins in

dwarf mice decreases significantly with age, whereas expression in controls holds constant or decreases slightly. Carbamoyl-phosphate synthetase, ASS1, and ARG1 exhibit elevated expression in the young dwarf mouse (22%, 41%, and 52%, respectively), but ultimately end up with expression levels at 33 months of age equal to (ASS1 and ARG1) or slightly higher than (CPS1 at 10% above) control mouse levels at 24 months.

The detoxification enzyme, glutathione S-transferase (GST) isoform $\mu 1$, displays increased expression in the dwarf mouse at 24 months compared to controls by 46% (Figure 4. E). In both dwarf and control, GST $\mu 1$ expression increases with age; however, in the dwarf mouse the expression at 33 months drops towards the levels measured in wild-type mice at 24 months. The oxidative defense enzyme, Cu,Zn-superoxide dismutase (SOD), is up-regulated 21 and 35% ($\sigma = \pm 3\%$ and $\pm 16\%$, respectively) in the dwarf at 24 and 33 months, respectively (Figure 4. F). Another oxidative defense enzyme, catalase, is slightly downregulated in the dwarf mouse from 2 to 24 months compared to same-age controls ($9\% \pm 6\%$ and $13\% \pm 1\%$), as well as decreasing in expression with age until 24 months (12% decrease, data not shown). Surprisingly, catalase expression in 33-month-old dwarfs increases to slightly above that in 24-month-old controls.

Western Blot Analysis

Western Blot analysis of four key proteins, ornithine decarboxylase (ODC), ARG1, GST $\mu 1$, and SOD was carried out. The latter three qualitatively confirm results of proteomic analysis, with the exception of ARG1 expression not increasing in the 33-month-old dwarf. Arginase 1 expression is significantly increased in the young dwarf compared to controls by 30%, followed by a drastic decrease in expression in the dwarf mouse at 24 months of age, with expression levels at 59% of those measured in the same-aged control. 33-month-old dwarfs have slightly lower ARG1 expression (~8%) than 24-month-old dwarfs. Ornithine decarboxylase, a protein expected to be down-regulated in the dwarf by biochemical pathway analysis and miRNA targeting, exhibits significantly decreased expression in the dwarf at both 24 and 33 months of age, compared to 2 and 24-month-old controls (Figure 5. A, B). Dwarf ODC1 expression at 2, 24 and 33 months is 75, 56 and 64%, respectively, of the values measured in 2-month-old controls. Similar, Dwarf ODC1 expression at 24 months is 82% that of the values measured in 24-month-old controls, and at 33 months is 94% that of 24-month-old controls ($p < 0.01$). GST $\mu 1$ is greatly over-expressed in 2- and 24-month-old dwarf mice (412% and 136% compared to same age controls, respectively); however, at 33 months of age the expression level drops drastically, to 76% of that in 24-month-old controls. Superoxide dismutase displays increased expression (~2X) in the dwarf mouse that decreases with age, whereas in the controls SOD expression increases with age—though never quite reaching dwarf levels (Densitometry in Supplemental Figure 2).

Statistical Correlation

Theoretical calculations reported by database algorithms of miRBase Targets Version 5 (<http://microrna.sanger.ac.uk/targets/v5/>) reveal strong correlations between the five qPCR-tested miRNAs and the respective target proteins (Table 2 and supplementary Table 1). Partial correlations calculated demonstrate significant correlation between experimental expression of miRNAs and target proteins (Table 3). All partial correlations have a calculated $|r|$ value greater than $|0.6|$.

Bioinformatic Analysis of Key microRNAs and their Candidate Target Proteins

Microarray analysis of miRNAs extracted from the livers of Ames dwarf and wild-type mice reveal strong correlations with the proteomic data from the same sample set (Table 1). The highest fold change detected by miRNA profiling between dwarf and control is seen with miR-27a. According to the miRBase Targets Version 5 database

(<http://microrna.sanger.ac.uk/>), both ODC1 and SRM key enzymes of polyamine biosynthesis, are targeted by miR-27a. Three other databases were employed with the hope to further confirm the predicted relationship between miR-27a and ODC1/SRM. Our searching results show that using the TargetScan, (http://www.targetscan.org/mmu_50/) database, miR-27a is reported to target the 3'-UTR of SRM but not of ODC1. The RNA22 database (http://cbcsrv.watson.ibm.com/rna22_targets.html) predicts the 3'-UTRs of both ODC1 and SRM to be targeted by miR-27a, with a stringent cutoff setup (maximum number of allowed UN-paired bases is 0 (ODC1)/ 2 (SRM) in seed/nucleus of 6 nucleotides, minimum number of paired-up bases in heteroduplex is 14; and the maximum folding energy for heteroduplex (Kcal/mol) is -25). No relation between miR-27a and ODC1/SRM is found in PicTar (<http://pictar.mdc-berlin.de/>) thus far (Supplemental table 2). This may be due to the fact that PicTar largely concentrates on human miRNA and its target prediction. Based on this exercise of algorithmic prediction, ODC1 and SRM were chosen for further functional studies as shown later in Figure 7 & Figure 8.

The relationship between miR-27a and ODC prompted us to examine further other candidate miR/target pairs. Towards this end, we performed comparative analysis of lead microRNAs selected by our array screening results with data obtained from Tandem Mass proteomic profiles. We found that, in addition to miR-27a, many other lead microRNAs are postulated to target key proteins of the urea cycle by gene family analysis, and furthermore their targets are not only identified by our proteomic profile results as lead proteins but also show inverse expression patterns (Table 1). For example, arginase 1 and ASS1 are each targeted by four different miRNAs (mmu-miRs-29b*, -676, -382*, & -669b target ARG1, and mmu-miRs-22, -127*, -470, & -411* target ASS1), all of which display relatively similar expression patterns and inverse correlations between miRNA and target gene expression. Expression levels of miR-669b increase with age in both mouse types; however, the dwarf increases at a much higher rate (2X that of the control) before leveling off between 24 and 33 months of age (Figure 2. Bvi, 3B, & 4C). MicroRNA-501-3p is postulated to target CPS1, and displays significantly increased expression over that of the controls (Figure 2. Bi, 3E, & 4A). Expression of miR-501-3p increases in the dwarf significantly at 33 months, while at the same time, CPS1 decreases to its lowest level. MicroRNA 96 targets GST μ 1, and shows decreased expression in dwarf mice, thus allowing for increased gene expression; however, expression in the dwarf at 33 months of age returns to levels similar to 24-month-old controls (Figure 3. D & Figure 4. E).

***In situ* detection of mmu-miR-27a and Immunostaining of ODC1 in wild type and dwarf mouse liver**

MicroRNA-27a expression patterns were further determined by *in situ* hybridization (ISH), using LNA (locked nucleic acid) probes (Figure 6). Hybridizations were carried out with liver sections from 2-month-old mice (both wild type control and dwarf mice). Consistent with our MMchip and qPCR validation results, we observed stronger *in situ* signals for miR-27a in dwarf than in wild type mouse livers (Figure 6, upper panels). ODC1, the predicted target of miR-27a, was detected by immunohistochemistry, as described in Materials and Methods; more ODC1 staining was observed in wild type than in dwarf mouse livers (Figure 6, lower panels). An antibody to SRM was not commercially available, and therefore the inverse relationship of miR-27a and its targets was evaluated solely with ODC1 for this assay, as well as for the transduced expression of this miRNA in the endogenous target study described in the following section. Taken together, the correlated inverse expression of miR-27a and its predicted target protein (ODC1) was confirmed *in situ*. Furthermore, these results provide additional verification that ODC1 is a candidate target of miR-27a repression at the post-transcriptional level.

Reporter functional assay of miR-27a with ODC1 and SRM

The 3'-UTRs of ODC1 and SRM were cloned and inserted into pHcRed1-C1 vectors, with the HcRed1 fluorescent protein (red fluorescence) as the reporter. Mouse miR-27a expression may be assayed by the transfected expression of green fluorescence (GFP) by a plasmid containing both mmu-miR-27a and GFP. To study the functional impact of miR-27a on the expression of either ODC1 or SRM, co-transfection experiments were carried out using miR-27a expression plasmids and either of the two cloned 3'-UTR reporter constructs. In co-transfected 293 and NIH/3T3 cells, we observed strong green and red fluorescence intensity at 24 hours after co-transfection, reflecting the input construct expression. Repression of the transfected miR-27a was observed beginning at 48 hours in 293 cells (Figure 7. A), and at 72 hours in NIH/3T3 cells (Figure 7B); this is evidenced by the reduced intensity of red fluorescence from miR-27a +ODC1 3'-UTR or +SRM 3'-UTR (Figure 7. A & B, Panel I, a-c and g-i). This reduction is not observed in the plasmid construct bearing scrambled sequences, instead of miR-27a, in either ODC1 3'-UTR or SRM-3'-UTR cells (Figure 7. A and B, Panel I, d-f and j-l), indicating that the expression of the red fluorescence protein is repressed by miR-27a *via* its binding to the 3'-UTRs of ODC1 or SRM. Due to the co-transfection regimen, transfection efficiency was not homogenous; only ~50% of the cell population showed positive inclusion. This prevented us from doing Western blotting; instead, we used quantitative image analysis to show the repression level by ratio comparison between transfected miR-27a and 3'-UTR constructs. As shown in Panel II.a, cells transfected with control plasmids containing the scrambled sequence exhibit no red fluorescence suppression via complementary binding to the ODC1-3'UTR; thus no difference was found between the intensities of the two fluorophores representing the two transfected plasmids. However, this was not the case when plasmids bearing the miR-27a insert were used. Here, the level of transfected ODC1-3'UTR intensity is significantly repressed to almost undetectable levels. Similar observations were seen with miR-27a/SRM and that of the scrambled control vector/SRM 3'-UTR (Panel II.b).

Next, endogenous ODC1 expression was measured in 293 and NIH/3T3 cells before and after miR-27a transfection. The results show that ODC1 expression indeed decreases in miR-27a-transfected 293 and NIH/3T3 cells, compared to scrambled control plasmid-transfected cells. Our transfection efficiency of ~50% provided the opportunity to study experimental *versus* control conditions by comparing fluorescence intensities among neighboring cells. For example, seen in the same field, the specific cell indicated by a double arrow with transfected miR-27a expression (Figure 8, Panel I. b) shows almost no detectable fluorescence intensity (Figure 8, Panel I. c). In contrast, the neighboring cell (indicated by a single arrow), which shows no transfected miR-27a expression, retains its endogenous ODC1 expression (Figure 8, Panel I.c). Similar differential fluorescence intensities between transfected cells and their endogenous ODC1 levels were not observed with plasmid constructs containing the scrambled sequence; scrambled sequence-transfected cells and their un-transfected neighbors show the same levels of fluorescence intensity. Again, we observed marked differences of fluorescence intensity between the transfected expression of miR-27a and endogenous ODC1 detected by antibody staining. In both cases, ODC1 is significantly lower than its normal level in miR-27a transfected cells, and not so in those carrying the scrambled sequence. This is particularly true in 293 cells, where endogenous ODC1 is almost undetectable in miR-27a-transfected cells (Figure 8 Panel I.c.). Thus, the specific repression by transfected miR-27a on endogenous ODC1 levels is indeed due to miRNA action, and not nonspecific binding *per se*. Similar observations were recorded when the same experiment was performed in NIH-3T3 cells (Figure 8, Panel I g-l). Quantitation of repression by transduced miR-27a was measured as described for Figure 7, A & B, Panel II, with 10 representative fields totaling more than 300 cells. Taken together, our results show that miR-27a represses ODC1 and SRM 3'-UTR reporters, and moreover,

endogenous ODC1 expression is similarly affected by transfection with this miRNA. Therefore, the relationship between miR-27a and ODC1/SRM is not only estimated by software analysis, but also validated in our experimental functional assays.

DISCUSSION

Most studies conducted to date on Ames dwarf mice and aging focus on growth hormone (GH), and more specifically, on the downstream reduction of the insulin-like growth factor (IGF-1), increased insulin sensitivity, and increased stress resistance and tumor suppression capabilities (Dominici 2002; Brown-Borg *et al.* 2005; Wang 2007; Wang *et al.* 2007; Brown-Borg 2009a; Brown-Borg 2009b). Most of these features are also noted in studies demonstrating that the Ames dwarf mouse's long-lived phenotype can be observed in altered GH, insulin/glucose metabolism, or IGF-1 by either gene deletion (*e.g.*, GH receptor knock out) or caloric restriction. Furthermore, increased longevity due to decreased GH or IGF-1 signaling led us to investigate the post-transcriptional regulation of genes involved in this desired phenotype—the delayed onset of aging—while bypassing undesirable effects associated with these hormonal deficiencies (*e.g.*, reduced size, impaired reproductive capacity, *etc.*). Our results show that control of expression of genes specifically involved in intermediate metabolism and toxin defense is altered early on in the dwarf mouse. Our identification of up-regulated key miRNAs, led by miR-27a, suggests that these miRNAs suppress protein expression to levels similar to those of the aged control, but with a nine-month delay.

At present, several databases with their particular algorithms are commonly used to predict miR/target pair relationships. However, target identification from these different algorithms may yield discordant results. For example, in our study, ODC1 is identified as a target for miR-27a by miRanda and RNA22, but not by the popular TargetScan program. Therefore, attempts to identify specific targets for a particular miRNA need to employ at least four programs (miRBase target version 5 (miRanda), TargetScan, RNA22, and PicTar) in the public domain, each with its own unique features. The first two databases are popular for miRNA target prediction. Targetscan considers the miRNAs sharing the same seed sequence as a family and presents the predicted target proteins for each of the miRNA families. However, this database does not incorporate rapidly updated miRNAs, as does miRanda. RNA22 is a powerful miRNA analysis algorithm with applicability similar to Targetscan as described above but with the additional utility for one to one prediction, *i.e.* one special interest miRNA for target proteins as we have presented in this report for miR-27a. Finally, Pictar is mainly providing the human and Drosophila miRNA target predictions. All four algorithms were used in this report and helped strengthen our hypothesis that protein members of the polyamine biosynthetic pathway were targeted by miR-27a (Supplemental table 2). Besides these programs available in the public domain, many personalized programs are also available in different bioinformatic laboratories; too many to be discussed here. Notwithstanding these programs and utilities, final determination of a particular miRNA/target relationship must be validated by functional studies, as shown in our results with miR-27a and its two targets, ODC1 and SRM, using reporter (Figure 7. A and B) and repression of endogenous gene expression assays (Figure 7. C).

The drastically reduced ornithine decarboxylase expression in dwarf mouse liver at 24 months of age, yet afterwards surprisingly increases towards the levels measured in aged controls (Figure 5. A and B) is very intriguing. Ornithine decarboxylase is the key rate-determining enzyme in polyamine biosynthesis, and catalyzes the reaction of L-ornithine to putrescine—a precursor of spermidine and spermine. MicroRNA-27a is implicated in a suite of studies pointing towards a role in cancer proliferation, transcription factor regulation, promotion of MDR1 expression, protective protein suppression and gastric adenocarcinoma,

and gastric mucosal atrophy (Arisawa *et al.* 2007; Mertens-Talcott *et al.* 2007; Liu *et al.* 2009; Wang *et al.* 2008; Zhu *et al.* 2008). In dwarf mouse liver, miR-27a expression increases with age, and is predicted to target ODC1 with significant complementary binding to the 3'-UTR of ODC1 mRNA and the 3'-UTR of SRM mRNA (the successive enzyme in the pathway to spermidine synthesis) (Supplemental Table 2). Our *in situ* and *in vitro* assays support this prediction, with ISH combined with immunohistochemical staining demonstrating an inverse expression disparity between miR-27a and ODC1 from wild-type controls (Figure 6). *In vitro* assays show that the 3'-UTRs of both ODC1 and SRM are apparently bound by miR-27a, and translation of the red fluorescent gene product is suppressed (Figure 7). *In vitro* endogenous expression assays show in two different cell types that ODC1 expression is reduced after transfection with the miR-27a vector, which is likely due to miR-27a binding to the 3'-UTR of endogenous ODC1 mRNA; thus inhibiting translation (Figure 8). Taken together, it is reasonable to suggest that polyamine synthesis in the dwarf mouse liver is posttranscriptionally suppressed *via* miR-27a.

Besides the essential function of polyamine biosynthesis in normal cell processes, increased polyamine biosynthesis resulting from an imbalance of arginine/ornithine metabolism or abnormal hormonal fluctuation has been linked to tumor cell proliferation, a variety of cancers, lesions, polyps and various other abnormal growths (Sogani 1972; Manteuffel-Cymborowska *et al.* 1995; Gritli-Linde 1997; Ignarro *et al.* 2001; Byun *et al.* 2008). Part of the ability of dwarf mice to suppress or avoid tumor or cancer growth may be attributed to the decreased polyamine biosynthesis resulting from reduced ARG1, ODC1, and possibly also SRM expression. Reduced expression of ODC1 could also be a result of the hormonal deficiencies in the dwarf mouse; ODC1 expression has been directly correlated with hormonal expressions of GH, PRL, testosterone, and estrogen (Gonzalez *et al.* 1991; Manteuffel-Cymborowska *et al.* 1995; Gritli-Linde 1997; Byun *et al.* 2008; Kondo *et al.* 2008). It is tempting to speculate that a relationship exists between hormone levels and miR-27a expression. What remains to be seen is why ODC1 expression increases in the aged dwarf (33-m.o.) towards the values measured in aged wild-type (24-m.o.) mice—correlating with sharply decreasing miR-27a expression detected by qPCR (Supplementary Figure 2A)—but with a presumed lack of hormonal stimulation. This may suggest that another factor is at play, working in coordination with miR-27a, ultimately allowing levels of ODC1 in the aged dwarf (33 m.o.) to reach levels observed in the aged control (24 m.o.). Further study using functional means (*e.g.*, knock-in and knock-out of miR-27a) may demonstrate that the observed expression contributes significantly to the dwarf's delayed aging.

Interestingly, CPS1, ASS1, and ARG1 exhibit increased protein expression in the young dwarf, followed by increasing miRNA suppression with age. This ultimately suppresses proteins to levels equal to or below those of older controls. It is well known that insulin represses transcriptional expression of proteins involved in urea synthesis, and many diabetes-associated complications arise from increased expression of ARG1 (Romero *et al.* 2008). The dwarf mouse, which exhibits reduced insulin levels compared to normal mice, would be expected to have over-expressed urea cycle proteins such as CPS1, ASS1 and ARG1; but because of miRNA regulation, CPS1 and ASS1 are either held slightly higher than, or suppressed to levels below, those of controls, depending upon the age examined. Unlike CPS1 and ASS1, ARG1 exhibits sharp miRNA suppression in Ames dwarf mice, to well below control levels, but then protein levels increase to reach—at 33 months of age—values equal to levels expressed in the 24-month-old control, thus displaying a 9 month lag before reaching parity with aged expression levels between dwarf and control, as also observed with ODC1. This pattern, occurring either fortuitously or because of dwarf physiology, should allow for adequate L-arginine production—a crucial substrate for nitric oxide production—while moderating its downstream use as a critical substrate of polyamine biosynthesis. Alternatively, the posttranscriptional suppression of these urea cycle proteins

may result from the reduced downstream expression—and thus reduced demand for the L-ornithine substrate—of critical polyamine biosynthesis proteins such as ODC1 and SRM.

A recent study shows that ODC1 activity and IGF-I and IGF-binding protein reductions result from dietary deficiencies of L-arginine (Cremades *et al.* 2004). Arginine can act as a secretagogue—a substance that promotes secretion of another substance—of GH and insulin in humans and other mammals (Rosenfeld *et al.* 1994; Morimoto *et al.* 2001). Taken together, these findings further emphasize a possible relationship between dwarf GH/IGF-1/insulin effects, miRNA regulation, and arginine/polyamine metabolism. Enhanced regulation of polyamine biosynthesis would be expected to contribute to the dwarf mouse's decreased incidence of cancer compared with wild-type mice (Ikeno *et al.* 2003).

The dwarf mouse is well known for its heightened oxidative and toxic defense capabilities. The marked over-expression of GST μ 1 in the dwarf mouse demonstrates its ability to conjugate toxic compounds with free glutathione (GSH) for removal. After embryonic development, GST μ 1 is continuously expressed throughout its life at levels higher than wild-type controls. A previous study on wild-type aging in mouse liver showed an increase with age in GST-targeting miRNA (Maes, *et al.*, 2008). However, in this study one GST-targeting miRNA, miR-96, displays significantly decreased expression in the dwarf mouse at 24 months of age, whereas the controls' expression remains relatively constant. At 33 months of age, however, miR-96 expression in the dwarf mouse returns to levels close to that of the wild-type, at the same time point that dwarf GST expression is similar to that of aged controls. Moreover, a previous study showed that dwarf mice display increased Cu,Zn-SOD and catalase activity in their youth compared to controls, but this activity declines with age (Hauck & Bartke 2000). In like manner, catalase expression decreased with age in our study; however, unlike in the previous study, it is expressed slightly less—instead of slightly more—than controls, and increases in expression at 33 months, although this extreme old age was not tested in the previous study. The increased expression of SOD and GST μ 1 in the young dwarf may suggest that enhanced oxidative and toxic defense in early life contributes to the delayed onset of aging observed with dwarf mice.

In conclusion, our study shows that miRNAs upregulated in dwarf mice correspond to alterations in target genes shown by our proteomic profiling, and reveal the level of posttranscriptional control that is presumably related to longevity determination. Specific miRNA upregulation associated with aging differs between Ames dwarf mice and their wild-type counterparts. This difference may well determine the slower rate of aging, since the dwarf seems to eventually arrive at proteomic expression levels similar to aged controls, but with a nine-month lag. Our work presented here implicates the function of miRNAs in delayed aging in the Ames dwarf mouse long-lived phenotype. Additionally, the prominent miR-27a and its reciprocal relationship with ODC1 expression, seen as early as 2 months of age, suggests a head-start in the dwarf mouse's ability to control the rate of intermediate metabolism signaling, and thereby potentially extend its life span. Future work with miRNAs, such as knock-in or knock-out transgenic mice with identified miRNAs such as miR-27a and its sisters, will reveal their functional impact in extending life span without hormonal deficiencies.

Experimental Procedures

Comparative proteomic and miRNA expression analysis were carried out in triplicate for each of 15 mice divided evenly into five categories: 2-month-old control, 2-month-old dwarf, 24-month-old control, 24-month-old dwarf, and 33-month-old dwarf. These ages represent mature, old, and extremely old age groups.

Mouse strain and samples

Male Ames dwarf mice and their wild-type siblings were produced in the Southern Illinois University animal facility. Tissue samples were collected from Ames dwarf and control mouse livers at 2, 24, and 33 (dwarf mice only) months of age. Comparative proteomic and miRNA expression analysis was carried out in triplicate for each of 15 mice divided evenly into five categories: 2-month-old control, 2-month-old dwarf, 24-month-old control, 24-month-old dwarf, and 33-month-old dwarf. Samples were rapidly frozen at -80°C until analysis. Tissue samples were processed as described below.

Total and small RNA extractions

Total RNA was extracted from frozen tissue blocks by grinding them in Trizol according to the manufacturer's instructions. Small RNA enrichment was carried out according to Park *et al.* (Park *et al.* 2002). Briefly, total RNA was adjusted to 400 μL with RNase-free water. Next, 50 μL of NaCl (5M) and 50 μL PEG 8000 (v/v 50%) were added. Samples were incubated on ice for 2 hours followed by centrifugation for 10 min. at 13,000 rpm at 4°C . The supernatant was transferred to a microcentrifuge tube, and 50 μL sodium acetate (3M, pH 4.6) and 1 mL of 100% ethanol were added. Samples were vortexed and incubated at -20°C for 2 h, then centrifuged at 12,000 g for 10 min. at 4°C . The supernatant was discarded, and 1 mL of cold 75% ethanol was used to wash the pellet. Samples were centrifuged again at 12,000 g (10 min, 4°C), the supernatant was discarded, and the RNA pellet was dried and dissolved in 12 μL of RNase-free water at 60°C for 10 min. Samples were quantified using spectrophotometry (260 nm), and stored at -80°C .

MicroRNA profiling

Samples of small RNA were labeled on their 3' end with digoxigenin (DIG) using the DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche Diagnostics, Indianapolis, IN). 1.0 μg of small RNA was labeled in a total volume of 20 μL , as described by Wang *et al.* (Wang *et al.* 2002). Mouse miRNA microarrays (MMChips) bore 367 anti-sense DNA sequences of mouse miRNAs obtained from miRBase (<http://microrna.sanger.ac.uk> Version 8.2). Hybridization and detection of miRNAs were carried out as previously described (Schipper *et al.* 2007).

Hybridization intensities were measured using an Expression 1680 scanner (Epson, Long Beach, CA); Array-Pro Analyzer 4.5 software (Media Cybernetics, Bethesda MD) was used for data acquisition. Whole cell area measurements were used to derive net intensity levels; the mean intensity of ring background around the spots was used for correction. Array-Pro Analyzer software was also used for normalization of MMChips, using mean signal intensity of all cells. SAM software, Version 3.02 (Significance Analysis of Microarrays, Stanford University, Stanford, CA) was used for microarray data analyses, including pairwise comparison (*T*-statistics) between strains and age groups from young to old. Kolmogorov-Smirnov statistics were generated using the Gene Set Enrichment Analysis (GSEA) software (Subramanian 2005). Unsupervised hierarchical clustering using Pearson's correlation was carried out with GenePattern software (www.broad.mit.edu/cancer/software/genepattern/; Broad Institute, Cambridge, MA).

qRT-PCR validation

QRT-PCR was carried out as described by Maes *et al.* (Maes, *et al.*, 2008). Briefly, 0.1 μg of small RNA was quantified using the NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) *via* real-time PCR. Mature DNA sense sequences (obtained from miRBase <http://microrna.sanger.ac.uk/>) were used as forward primers. Validation miRNAs were chosen, one from each postulated target gene. MicroRNA primer sequences used were

mmu-miR-27a (ttcacagtggctaagttccgc), mmu-miR-669b (agttttgtgtgcatgtgcatgt), mmu-miR-22 (aagctgccagttgaagaactgt), mmu-miR-96 (tttggcactagcacatttttgc), and mmu-miR-501-3p (aatgcacccgggcaaggatttg). As a reference sequence, 5S rRNA was used, probed using an internal forward primer (cagggtcgggcctggttagtacttg). MicroRNA expression fold changes between ages were calculated using the delta Ct method, relative to controls following normalization with levels of 5S rRNA.

Protein extraction

To obtain total protein, tissues were diced, then homogenized in 2 volumes (g/mL) of RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, pH 8.0) containing 1 × Protease Inhibitor (Calbiochem, San Diego, CA) and centrifuged at 10,000 × g for 10 minutes; the supernatant was harvested. One volume of RIPA buffer was added to the pellet and sonicated 3 × 10 s, centrifuged, and supernatants pooled. The Bradford method was used to quantify total protein concentration with BioRad reagents (BioRad, Hercules, CA).

Western Blot Analysis

Total protein was resolved *via* SDS-PAGE, and blotted on nitrocellulose membrane Protran BA 85 (Whatman Schleicher & Schuell, Springfield Mill, Maidstone, Kent, UK) as previously described (Sarojini *et al.* 2007). Rabbit anti-Ornithine Decarboxylase (ODC1), anti-Arginase 1 (ARG1), and goat anti-Argininosuccinate synthetase (ASS1) primary antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA), rabbit anti-Vimentin (normalization control) was produced in-house, and anti-Glutathione S-transferase μ 1 (GSTM) was purchased from Aviva Systems Biology (San Diego, CA). Secondary antibodies used were either sheep anti-rabbit horseradish peroxidase (HRP) or donkey anti-goat HRP, obtained from Santa Cruz Biotechnologies. Films were developed in a dark room, and proteins were quantified using densitometry software (ImageQuant version 5.2, Molecular Dynamics, www.mdyn.com), using vimentin as a normalization control. Three samples for each group were detected and analyzed.

Proteomics

Total protein (150 μ g) was precipitated with 6 volumes of cold acetone (-20°C), then allowed to decant at -20°C . Proteins were denatured, reduced, and alkylated according to manufacturer's instructions (iTRAQTM labeling kit, Applied Biosystems). Samples were Trypsin-digested (Trypsin from Promega, Madison, WI) for 16h at 37°C . Mouse peptides of control 2 months, dwarf 2 months, control 24 months, and dwarf 24 months were labeled with iTRAQ reagents 114, 115, 116, & 117, respectively. Labeled peptides were then identified and quantified via tandem mass spectrometry. Because the limitation of the quadruplex labeling, the 33-month proteomic was not performed.

Fractionation of peptides was performed on a cation PolySulfoethyl A column (4.6 nm × 20 cm, PolyLC Inc., Columbia, MD), using a BioCAD workstation (Applied Biosystems) as described by Cong *et al.* (Cong *et al.* 2006). C18 ZipTips (Millipore Corporation, Billerica, MA) were used to desalinate fractions prior to LC-MS/MS. A QSTARTTM XL hybrid liquid tandem mass spectrometry (LC-MS/MS) system (Applied Biosystems), interfaced with an 1100 Series Capillary LC system (Agilent, Sta. Clara, CA) with an analytical Vydac HPLC Column (75 μm × 150 mm; Vydac MS C18 300A, Alltech Associates Inc., Nicholasville, KY) was used for peptide fractionation and identification. MS TOF scans were acquired from m/z 350 to 1600, with up to two precursors selected for MS/MS from m/z 60 – 2000, using information-dependent acquisition and rolling collision energy applied to promote fragmentation.

Nanospray MS and MS/MS data were analyzed using ProteinPilot™ Software 2.0.1 (Applied Biosystems) for iTRAQ identification and quantification between samples. N—termini, Lys, Tyr, and Cys modifications were selected as fixed, Met oxidation as variable, one missed cleavage allowed, precursor error tolerance was set at <0.15 Da, and product ion error tolerance at <0.1 Da. For quantification of iTRAQ labeled peptides between age groups, the cutoff was set at >95%, with a ProtScore threshold of 1.30.

Data mining and statistic analysis for candidate targets of lead miRNAs

MicroRNA target predictions were acquired and downloaded from the miRBase website <http://microrna.sanger.ac.uk/> (Griffiths-Jones 2008). Each target prediction was inversely correlated with proteomic and gene expression data. Three other databases, TargetScan (http://www.targetscan.org/mmu_50/), RNA22 (http://cbcsrv.watson.ibm.com/rna22_targets.html) and PicTar (<http://pictar.mdc-berlin.de/>) were used for selected miRNAs and their targets. For RNA22, a stringency cutoff was set up with the following parameters: the maximum number of allowed UN-paired bases was 0 in a seed/nucleus of 6 nucleotides, the minimum number of paired-up bases in heteroduplex was 14; and the maximum folding energy for a heteroduplex was −25 Kcal/mol.

Predictions of microRNA/gene target correlations were assessed on the following three levels: 1) significance of experimentally determined expression changes (p-value and q-value %); 2) probability of microRNA and target gene relationship; and 3) correlation between miRNA expression and gene expression changes (Partial correlations).

On the first level, the microarray data were processed using SAM software, Version 3.02, which yields fold change comparisons and a q-value percentage to demonstrate the confidence of a real expression difference. To supplement and confirm the microarray analysis, qPCR was carried out in triplicate, with the single best candidate miRNAs — determined by *level two* analyses, described below — that exhibited significant variation in microarray analysis, and experimentally determined significant inverse expression with its predicted target protein. Target protein expression was determined using global proteomic profiling, as specified above, with greater than 95% confidence (p-value reported in Table 1), and confirmed *via* Western blot analysis on a few select proteins.

On the second level of statistical analysis, the miRBase Targets Version 5 and miRanda algorithms were employed to demonstrate a high theoretical correlation between the selected miRNAs and their target proteins. These correlations are reported in Table 2. According to John *et al.*, the percentage of false positives is 30%, 24%, 19% and 9% for transcripts with 3, 4, 5 and 10 target sites (John *et al.* 2004). These calculations are based on running real miRNA sequences that were randomly shuffled through the same predictive algorithms to measure their predicted targeting potential. The results of this exercise were compared to the results of the real miRNA targeting calculations. All of the miRNA/targets listed in our tables have greater than 10 target sites. *P* values given are the best *P* value of the selected miRNA for a transcript.

As described above, besides miRanda algorithms, we also used the TargetScan, RNA22 and PicTar to do the relation prediction between miR-27a and ODC1/SRM (supplemental table 2). In the TargetScan database, for each input protein, the list of miRNA families will be demonstrated with the target sites. For specific sequences that are matched between miR-27a and ODC1 or SRM, we used RNA22. In other words, we loaded the sequence of mmu-miR-27a and 3'-UTR sequences of ODC1 and SRM (sequences obtained from the NCBI genebank) to cover all the possible target sites as revealed by RNA22. PicTar database is provided publicly for human and Drosophila miRNA target predictions. Like miRanda, by

inputting the miRNA name, it will list all the targets. Since the current study is for mouse microRNAs, we only used it for a supporting searching in addition to the first 3 databases.

The third level of statistical correlation is a direct linear regression using ANOVA statistical analysis (*via* SPSS software version 13.0), to calculate partial correlation coefficients between the five miRNAs and their corresponding predicted target gene expressions, obtained experimentally *via* qPCR, MMChip and Western Blot, or proteomics, respectively (ASS1 and CPS1 did not undergo Western Blot analysis). The three input variables were miRNA expression change, target gene expression change, and age of mouse sample.

For all above tasks, attribution for genes and proteins to a particular gene family was made according to the SOURCE (<http://source.stanford.edu>) and BRENDA (<http://brenda-enzyme.info>) databases.

***In situ* hybridization with mmu-miR-27a locked-nucleic acid (LNA) probe**

MicroRNA 27a *in situ* hybridization followed the protocol described by Obernosterer *et al.* (Obernosterer *et al.* 2007). Wild type mouse liver and dwarf mouse liver tissue (2-month-old) were collected and fixed in 4% paraformaldehyde for 2 hrs at 4 °C. Tissues were kept in PBS with 30% sucrose overnight at 4 °C, frozen in Tek O.C.T. (Sakura Finetek, CA) on dry ice, and sectioned at 10 µm on the cryostat (Leica, Germany). In brief, tissue sections were washed in PBS for 10 minutes, then placed in Acetylation solution (98% DEPC, 1.3% triethanolamine (Fluka, St. Louis, MO), 0.175% HCl, 12% acetic acid (Sigma, St. Louis, MO) for 20 min. Next, the sections were digested by Proteinase K (25 µg/mL, Sigma P2308) for 5 min at room temperature, washed in PBS for 5 minutes, and prehybridized at 50 °C for 4 hrs. The mmu-miR-27a LNA probe bearing an embedded Digoxigenin (DIG) sequence was purchased from Exiqon (Woburn, MA). Probes (1 nM) were denatured with denaturing hybridization solution at 95 °C for 5 min, then added to the slides and hybridized at 50 °C overnight. The next day, the slides were washed in 5 × SSC at 60 °C for 5 min, and 0.2 × SSC 60 °C for 60 min. After blocking for 1 hr (2% FCS), sections were incubated with anti-DIG antibody (Roche, Indianapolis, IN, 1: 2000) overnight at 4 °C. Localization of the DIG antibody-labeling was performed by further reaction *via* NBT (Nitro blue tetrazolium chloride)/BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) for color development 1 or 2 days at room temperature. The images were examined on a Zeiss fluorescence microscope (Carl Zeiss, Brighton, MI) and AxioVision Rel. 4.6 imaging system.

ODC1 Immunohistochemical staining

Sections of the same frozen tissue specimens used above for the *in situ* hybridization studies were blocked and incubated with ODC1 antibodies (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Alexa 594 goat anti-rabbit IgG (1:400; Invitrogen) was incubated with sections for 40 min at 37 °C. The images were examined on a Zeiss fluorescence microscope (Carl Zeiss, Brighton, MI) and AxioVision Rel. 4.6 imaging system.

Mouse miR-27a preparation and the 3'-UTRs of ODC1 and SRM cloning

Mouse miR-27a expression clone and scrambled control were purchased from GeneCopoeia (Germantown, MD). The 3'-UTRs of mouse ODC1 and SRM were amplified from mouse genomic DNA (727 bp and 337 bp, respectively). The amplified sequence for the 3'-UTR of ODC1 is from 2 to 728 bp downstream of the stop codon, lacking the 25 bp upstream of the 3'-terminal of UCSC gene uc007ncv.1. The amplified sequence for the 3'-UTR of SRM is from 23 bp upstream to 311 bp downstream of the stop codon, which is 1 bp upstream of the 3'-terminal of UCSC gene uc008vuw.1. According to miRanda database (Supplemental

Table 2), miR-27a target sites are located at bp positions 287–307 \ and bp positions 26–44 downstream of the stop codon in the final reporter clones for 3'-UTRs of ODC1 and SRM respectively. The following primers were used for cloning:

ODC1-EcoRI-5.1, 5'-GAATTCTTAATGCCATTCTTGTAGCTCTTGC-3',

ODC1-BamHI-3.1, 5'-GGATCCGGAAGTTGACTGCCGATGTT-3',

SRM-EcoRI-5.1, 5'-GAATTCTTAAGGAAGGCCCTCAATGACATA-3'

SRM-BamHI-3.1, 5'-GGATCCCAGAGGTCATGACTGAGCTTGT-3'

(Underlined: restriction site, bold: in-frame stop codon)

The amplified DNA fragments were cloned into the pDrive cloning vector (Qiagen, Valencia, CA), then subcloned into pHcRed1-C1 vector (Clontech, Mountain View, CA) using EcoRI and BamHI sites. All constructs were confirmed by DNA sequencing.

Cell transfection/co-transfection

HEK 293 and NIH/3T3 cells at 70–80% confluence (ATCC, Manassas, VA) were used for transfection experiments with the cell line nucleofector kit V (Lonza Walkersville Inc., Walkersville, MD). Briefly, the cells were kept in culture for 48 hrs, trypsinized and collected. 1×10^6 cells were resuspended in 100 μ l nucleofector solution. 5 μ g of plasmid was loaded into a cuvette for each transfection/cotransfection. The experimental groups included: control, miR-27a, miR-27a+ ODC1 3'-UTR, miR-27a+ SRM 3'-UTR, control+ ODC1 3'-UTR, and control+ SRM 3'-UTR. Program Q-001 or A-033 was applied for HEK 293 cells or mouse NIH/3T3 cells, respectively. The cells were then immediately plated out in pre-warmed medium, supplemented with 10% FBS, into 35 mm glass bottom Petri dishes (MatTek, Ashland, MA). 72 hrs (for 293 cells) or 48 hrs (for NIH/3T3 cells) later, the cells were fixed with 4% paraformaldehyde in PBS, and imaged on a Zeiss fluorescence microscope (Carl Zeiss, Brighton, MI) and AxioVision Rel. 4.6 imaging system. For the endogenous ODC1 detection, scrambled control or miR-27a-transfected 293 and 3T3 cells were fixed and incubated with ODC1 antibody and Alexa 594 goat anti-rabbit, as described above.

In general, the transfection efficiency is ~50% in all our experiments; therefore, we were not able to perform grind-and-find experiments to examine the transfected gene's impact on its targets in terms of the expression levels of either endogenous target, ODC1-protein, or co-transfected 3'-UTR constructs, *e. g.* ODC or SRM 3'-UTRs. (Endogenous SRM was not examined, because no commercial antibody to this protein is available for immunocytochemistry.) To resolve this problem, the putative reciprocal expression between the expression of transduced miR-27a and that of endogenous ODC1 or co-transfected 3'-UTR constructs was determined *via* quantitation of respective intensities between the former and latter by measurements of individual cells—green *versus* red fluorescence intensities, or green *versus* ODC antibody staining intensities. Individual cell intensity was generated in digitized output by tracing the cell circumference; the final measurement was obtained using densitometry software (ImageQuant version 5.2, Molecular Dynamics, www.mdyn.com). Fluorescence intensity of individual cells was obtained by this method; for each experimental condition described above, ten separate fields of ~30 cells were measured, and their digitized numerical values were recorded and used for statistical analysis for significance. The results are presented in the histograms shown in right panels of Figure 7A, 7B and Figure 8, with photographic images showing the represented fields, as in the left panels of the above figures, used for the quantitation of the fluorescence images.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Heat map demonstration of expression levels with respect to age and mouse category (control, C or dwarf, D). Intensity of color equals relative expression, with blue representing decreasing expression, and red representing increasing expression. A.) Expression comparison of dwarf *versus* control at 2 months of age, B.) 24 months, C.) 24 month-old control *vs.* 24- and 33-month-old dwarf. Values are averages of 3 mice per age group and category.

Figure 2.

A.) Graphical representation of miRNA, mmu-miR-27a, which exhibits the greatest fold change in dwarf mice *versus* wild-type controls. B.) Graphical representation of key miRNA expression of dwarf mouse miRNA *versus* control. Samples are represented by age in months (2, 24, or 33) and C or D (control or dwarf mouse, respectively).

Figure 3. Quantitative Real-Time PCR (qRT-PCR) confirmation of MMChip results. A) Predicted target of ODC, microRNA mmu-miR-27a, which displays maximum increased fold change; B) predicted target of ASS1, mmu-miR-22; C) a postulated target of ARG1, mmu-miR-669b; D) postulated target of GST μ 1, mmu-miR-96; and E) postulated target of CPS1, mmu-miR-501-3p. The C point on the x-axis represents C24 for the red line, or C2 for the blue line. (Error bars = Standard Deviation, [σ]).

Figure 4.

Graphical representation of protein expression in dwarf mice *versus* controls. Samples are represented by age in months (2, 24, or 33) and C or D (control or dwarf mouse, respectively). The C point on the x-axis represents C24 for the red columns, or C2 for the blue columns.

Figure 5.

A) Western Blot analysis of ornithine decarboxylase (ODC1), Arginase 1 (ARG1), glutathione S-transferase (GST) μ 1, superoxide dismutase (SOD), and Vimentin (VIM) as normalization control. (Histogram of densitometry is in Supplementary Figure 2).

B) Histogram of the average densitometry measurements of ODC1 (error bars $\pm \sigma$, ** $p < 0.01$).

Figure 6.

In situ hybridization (ISH) of mmu-miR-27a (upper panels) and immunohistochemical staining of ODC1 (lower panels). Increased hybridization of mmu-miR-27a is observed in dwarf liver tissue (middle upper panel) *versus* control mouse tissue (left upper control), whereas decreased ODC1 staining is observed in the dwarf tissue (middle lower panel) *versus* control mouse tissue (left lower control).

Figure 7.

A) Panel I showing phase contrast (a, d, g, & j) and green (b, e, h, & k) and red fluorescence (c, f, i, & l) images of the same field. The miR-27a/ODC1 cotransfected 293 cells express

reduced red fluorescence, which shows that the 3'-UTR of ODC1 is repressed by the input miRNA and this is not true for the red fluorescence from the scrambled control/ODC1 cotransfected cells. It demonstrates that the miR-27a can indeed suppress the target protein (red fluorescence protein) through 3'-UTR of ODC1 (Panels a–c). This effect is absent when a plasmid carrying a scrambled sequence is used (Panels d–f). Similar experiments were performed with plasmids containing the 3'-UTR of SRM, as shown in Panel I g–I, with the plasmid carrying a scrambled sequence as control (j–l). **Panel II:** quantification of fluorescence in co-transfected cells (ODC1 3'UTR: upper, SRM 3'UTR: lower). Details on the quantitation of fluorescence intensities are included in Materials and Methods. The FITC and TRITC filter sets were employed to detect the green and red fluorescence of miR-27/scrambled control expression plasmid and 3'-UTR reporter plasmids.

B.) Panel I showing phase contrast (a, d, g, & j) and green (b, e, h, & k) and red fluorescence (c, f, i, & l) images of the same field. The miR-27a/ODC1 cotransfected NIH-3T3 cells express significantly reduced red fluorescence than from that of the scrambled control/ODC1 cotransfected cells, which demonstrates that the miR-27a can indeed suppress the target protein (red fluorescence protein) via binding to the 3'-UTR of ODC1 (Panel a–c); an event not seen with the plasmid carrying a scrambled sequence is used (Panel d–f). Similar experiments were performed with plasmids containing 3'-UTR of SRM, as shown in Panel I g–I, with the plasmid carrying a scrambled sequence as control (j–l). **Panel II:** quantification of the fluorescence in co-transfected cells (ODC1 3'UTR: upper, SRM 3'UTR: lower). Details on the quantitation of fluorescence intensities are included in Materials and Methods. The FITC and TRITC filter sets were employed to detect the green and red fluorescence of miR-27/scrambled control expression plasmid and 3'-UTR reporter plasmids.

Figure 8.

Panel I: Immunocytochemistry analysis of miR-27a suppression of endogenous ODC1 in both 293 (a to f) and NIH-3T3 cell strains (g to l). The cells shown with phase contrast (a, d, g, j), plasmid transfected (green fluorescence; b, e, h, k) and ODC1 immunostaining (red fluorescence; c, f, i, l) are from the respective identical fields. In each set (293 and NIH-3T3 cells), miR-27a transfected cells with double arrows (b and h) have dramatic ODC1 staining reduction (c and i) compared to ODC1 intensity in neighboring cells lacking miR-27a transfection (c and i; with single arrow). This pattern does not pertain for scrambled plasmid-transfected 293 or NIH-3T3 cells (e, f, k, l). Scrambled plasmid-transfected cells (with double arrows in e and k) show ODC1 staining intensity identical to un-transfected cells (single arrow) (e, f, k, l). **Panel II:** quantification of the fluorescence for plasmid transfection and ODC1 staining. (293 cells: upper, NIH-3T3 cells: lower). Details on the quantitation of fluorescence intensities are included in Materials and Methods. The FITC and TRITC filter sets were employed to detect the green and red fluorescence of miR-27/scrambled control expression plasmid and 3'-UTR reporter plasmids.

Figure 9.

Urea cycle/ornithine metabolism pathway diagram, showing proteins and the miRNAs that are postulated to posttranscriptionally regulate target protein expression. In order of the cycling pathway: 1) Carbamoyl-phosphate synthase (CPS1), 2) Ornithine carbamoyltransferase (OCT), 3) Argininosuccinate synthase 1 (ASS1), 4) Argininosuccinate lyase (ASL), 5) Arginase (ARG), 6) Nitric oxide synthase (NOS), 7) Ornithine decarboxylase (ODC), and 8) Ornithine aminotransferase (OAT).

Table 1

Fold changes of microRNA and protein expression

Fold change comparison of miRNA and protein expression levels. MicroRNAs are listed above their predicted target protein (leftmost column). Values represent average fold changes in 3 mice from each of five categories: control 2-month-old (C2), dwarf 2-month-old (D2), control 24-month-old (C24), dwarf 24-month-old (D24), and dwarf 33-month-old (D33). Standard deviations for protein expression comparison are listed under fold change values.

microRNA & Target protein		vs. 2-month-old control				vs. 24-month-old control			
		C24 (q-value%)	D2 (q-value%)	D24 (q-value%)	D33 (q-value%)	D2 (q-value%)	D24 (q-value%)	D33 (q-value%)	
						</			

	vs. 2-month-old control				vs. 24-month-old control		
<i>microRNA</i> &							
Target protein	C24 (<i>q-value</i> %)	D2 (<i>q-value</i> %)	D24 (<i>q-value</i> %)	D33 (<i>q-value</i> %)	D2 (<i>q-value</i> %)	D24 (<i>q-value</i> %)	D33 (<i>q-value</i> %)
<i>mmu-miR-96</i>	0.95 (>10)	0.70 (9.52)	0.90 (>10)	1.32 (0.0)	0.74 (>10)	1.14 (>10)	1.37 (0.0)
GST μ 1 (<i>p-value</i> = 0.01)	1.32 ($\sigma \pm 1.10$)	1.28 ($\sigma \pm 0.22$)	1.95 ($\sigma \pm 0.34$)	1.57 ($\sigma \pm 0.12$)	0.97 ($\sigma \pm 0.16$)	1.46 ($\sigma \pm 0.13$)	1.19 ($\sigma \pm 0.04$)

σ = Standard Deviation

Table 2

MicroRNA target predictions via miRBase

Table of microRNA target gene predictions. The computed data of each miRNA/predicted target gene pair may be used to estimate the false positive rate (FDR) of miRNA/target relations. Total sites means the total number of conserved targets found in a transcript. MicroRNAs highlighted in gray were used for qPCR confirmation.

miRs	Target Gene Name	P-value	UTR Length (bps)	Total Sites	No. miRNAs predicted to target
mmu-miR-27a	Odc1	0.0428941	333	17	49 [+]
	Srm	0.0150736	312	15	56 [+]
mmu-miR-669b	Arg1	0.000104109	389	19	41 [+]
mmu-miR-22	Ass1	0.0225366	255	11	29 [+]
mmu-miR-96	Gstm1	0.0298408	431	13	36 [+]
mmu-miR-501	Cps1	0.018076	1000	12	16 [+]
mmu-miR-127*	Ass1	0.0266144	255	11	29 [+]
mmu-miR-411*	Ass1	0.0315475	255	11	29 [+]
mmu-miR-470	Ass1	0.0372748	255	11	29 [+]
mmu-miR-29b*	Arg1	0.00169758	389	19	41 [+]
mmu-miR-382*	Arg1	0.0316054	389	19	41 [+]
mmu-miR-676	Arg1	0.0453475	389	19	41 [+]

Table 3
Statistical correlation between miRNA and predicted target gene expression

Linear regression analysis, demonstrating correlation between miRNAs and their respective predicted target gene expression, determined by qPCR and Western Blot or proteomic profiling, respectively.

MicroRNA and target gene	r	p-value
miR-27a qPCR/Odc1 Western blot	−0.683	0.074
miR-669b qPCR/Arg1 Western blot	−0.752	0.001
miR-96 qPCR/Gstm1 Western blot	−0.776	0.001
miR-22 MMchip/Ass1 proteomic	−0.82	0.001
miR-501-3p MMchip/CPS1 proteomic	−0.662	0.009