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Post-transcriptional regulation of IGF1R by key microRNAs in long–lived mutant mice

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Summary

Long-lived mutant mice, both Ames dwarf and growth hormone receptor gene–disrupted or knockout strains, exhibit heightened cognitive robustness and altered IGF1 signaling in the brain. Here, we report, in both these long-lived mice, that three up-regulated lead microRNAs, miR-470, miR-669b, and miR-681, are involved in posttranscriptional regulation of genes pertinent to growth hormone/IGF1 signaling. All three are most prominently localized in the hippocampus and correspond to reduced expression of key IGF1 signaling genes: IGF1, IGF1R, and PI3 kinase. The decline in these genes' expression translates into decreased phosphorylation of downstream molecules AKT and FoxO3a. Cultures transfected with either miR-470, miR-669b, or miR-681 show repressed endogenous expression of all three genes of the IGF1 signaling axis, most significantly IGF1R, while other similarly up-regulated microRNAs, including let-7g and miR-509, do not induce the same levels of repression. Transduction study in IGF1-responsive cell cultures shows significantly reduced IGF1R expression, and AKT to some extent, most notably by miR-681. This is accompanied by decreased levels of downstream phosphorylated forms of AKT and FoxO3a upon IGF1 stimulation. Suppression of IGF1R by the three microRNAs is further validated by IGF1R 3′UTR reporter assays. Taken together, our results suggest that miR-470, miR-669b, and miR-681 are all functionally able to suppress IGF1R and AKT, two upstream genes controlling FoxO3a phosphorylation status. Their up-regulation in growth hormone signaling-deficient mutant mouse brain suggests reduced IGF1 signaling at the posttranscriptional level, for numerous gains of neuronal function in these long-lived mice.

Key words: microRNA; aging; IGF1; IGF1R; growth hormone; Ames dwarf mice and GHRKO mice; miR-470; miR-669b; miR-681; cognitive robustness and longevity.

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Introduction

In the past decade, the insulin-like growth factor (IGF) axis of the somatotrophic signaling pathway has emerged as a major determining factor of longevity in mice; a parallel regulatory molecular mechanism is seen in evolutionarily distant species, including *Caenorhabditis elegans* and fruit flies (Rozing *et al.*, 2009). Consistently, in every long-lived organism from worm to mouse, a common theme in regulation of life span is the loss of function of IGF signaling, precipitating an extension of life span (Bartke, 2011). In mice, pulsate bursts of growth hormone (GH) from the anterior pituitary gland ensure GH biological activity, which involves binding to the GH receptor, leading to the synthesis and secretion of IGF1 (Le Roith *et al.*, 2001). IGF1-induced intracellular signaling may be a prototypical example of the antagonistic pleiotropy paradigm of aging: genes needed early in life may be deleterious after reproduction or in old age (Aleman & Torres-Aleman, 2009), as paradoxically, IGF signaling is necessary to promote growth and differentiation during development, yet its reduced activity is associated with life span extension in a wide variety of animals, and even in long-lived humans (Bartke, 2011). Therefore, the switch from the developmental need for IGF action to reduced activity in adult life becomes a key molecular regulator of life span determination, providing specific control of GH-induced effects, and impacts various signaling pathways.

The Ames dwarf mouse has a mutation in the *prop-1* locus, leading to GH, prolactin, and thyroid-stimulating hormone deficiencies (Sornson *et al.*, 1996). These mutants are hypersensitive to insulin, maintaining lower fasting glucose concentrations (Dominici *et al.*, 2002). However, the impact of GH loss in Ames dwarves, intertwined with the loss of the other two hormones, raises the need for a much cleaner mouse model; this is fulfilled by a mouse strain in which the GHR/binding protein gene has been disrupted [(growth hormone receptor gene–disrupted or knockout (GHRKO)] (Zhou *et al.*, 1997). This mouse is long-lived (Coschigano *et al.*, 2000), and similar phenotypically and physiologically to the Ames dwarf, but without the complications of prolactin and thyrotropin deficiencies (Hauck & Bartke, 2000). Thus, these long-lived dwarf mice share as common characteristics GH signaling deficiency, low circulating IGF1, and high insulin sensitivity.

Because of pioneering work on allele deletion from *C. elegans* to hormone-deficient mutant mice, the loss of IGF1 signaling and its impact on longevity have become central themes of longevity studies (Flurkey *et al.*, 2001); a current trend studies their and their outcome impact on metabolism through the target of rapamycin (TOR) signaling (Martin & Hall, 2005). The cross-talk between GH and IGF1 creates fascinating complexities, linking many intriguing functions of dozens or hundreds of networks in the organismic determination of life span (Dominici *et al.*, 2005). Despite this complex scenario, a central axis remains at the core of the intertwining networks, that is, from *C. elegans* to mice, IGF1 binds to IGF1R, activating intracellular kinases like phosphatidylinositol-3 kinase (PI3K), which in turn facilitates phosphorylation of protein kinase B (PKB/Akt) (Cantley, 2002; Vivanco & Sawyers, 2002). This activated PI3K/Akt pathway changes the phosphorylation status of a subgroup of the Forkhead family of transcription factors (FoxO proteins) that are instrumental in regulating genes involved in apoptosis, metabo-

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lism, and cell cycle process (Tran *et al.*, 2003). Akt phosphorylates transcription factor FoxO3a, enforcing its cytoplasmic localization and thus preventing its nuclear translocation, and truncating its transcriptional activation of hundreds of genes (Brunet *et al.*, 1999). An interesting facet of the prolongevity function of nuclear FoxO3a is its counteracting role in reducing oxidative stress by activating transcription of antioxidant enzymes such as mitochondrial superoxide dismutase (MnSOD) and catalase.

The amazing parallel life span extension between mutant mice with hormonal deficiency and *C. elegans* via genetic mutation led us to realize that levels of control of longevity could be achieved without the gross knockdown of one allele of members of the IGF1 signaling pathway. However, a missing link in our understanding of GH-induced signaling deficiency, and reduced signaling of the cascade of molecular events described earlier, is how the loss of function actually occurs, and what are the pivotal factors switching genes to a lower activity mode. In *C. elegans*, modulation of IGF1 signaling is widely reported to be regulated by various molecular factors that maintain equilibrium between aging and anti-aging, either dependent upon or independent of FoxO3a transcription factor activity (Gems & McElwee, 2003; McElwee *et al.*, 2006; Ayyadevara *et al.*, 2009; Shmookler Reis *et al.*, 2011). Not surprisingly, the role of microRNAs (miRNAs) in regulation of GH/IGF signaling has been reported recently (Yu *et al.*, 2008; Maes *et al.*, 2009; Shan *et al.*, 2009), controlling the equilibrium between expression of these noncoding RNAs and the levels of their target gene expressions. We and others have reported that profiles of miRNA expression change during aging, and their target genes are involved in many vital cellular functions; this is well documented in degenerative processes during normal aging (Liang *et al.*, 2009; Maes *et al.*, 2009; Bates *et al.*, 2010; Lanceta *et al.*, 2010; Khanna *et al.*, 2011; Li *et al.*, 2011a,b). Conceptually, the intermediate regulatory steps between reduced GH signaling and its impact on the expression of genes involved in the IGF1 axis are best accomplished via one or more families of molecular factors with efficient and versatile modes of operation. As reported earlier by our group, increased miR-27a expression in Ames dwarf mouse liver represses genes involved in oncogenesis, associated with the reduction of tumor incidence in Dwarf mice (Bates *et al.*, 2010). The present paper is the sequel to this paper on Ames dwarf mouse liver, aiming to unravel the miRNA-directed regulation of the other phenotype associated with long-lived mice, that is, the gain of neuronal functionality, mainly manifest in cognitive robustness.

In this report, we include GHRKO mutant mice, not only because of the direct impact on the IGF1 pathway of specific GH signaling deficiency but also for comparative analysis of parallel miRNA-directed regulation in Ames dwarf mutants. Using an in-house-designed miRNA microarray chip assay (MM chips), we identified over-expression of five miRNAs (miR-470, miR-669b, miR-681, miR-501-5p, and let-7g) in Ames dwarf mouse brain, validated by quantitative PCR (qPCR) and *in situ* hybridization (ISH) in brain tissues for the first three miRNAs. Quantitative PCR and ISH studies show that miR-470, miR-669b, and miR-681 are up-regulated in the GHRKO brain as well. Increase in these three miRNA expressions corresponds to decreases in IGF1, IGF1R, and PI3 kinase, and the phosphorylated forms of their downstream members, AKT and FoxO3a in both mutants. Transfection study shows that miR-470, miR-669b, or miR-681 represses IGF1R more significantly than the other related proteins; this is further validated by their individual capability to repress the expression of the reporter construct of this gene's 3′-untranslated region (3′UTR). Finally, the direct impact of each of these three miRNAs on IGF1R and other related genes of this signaling pathway was tested by IGF1 stimulation in IGF1-responsive cultures expressing any of the three miRNAs. Our results show that upon stimulation with IGF1, miRNA-over-expressing cultures exhibit repressed expression primarily of IGF1R, with some degree of repression of AKT as well, most notably by miR-681. Most importantly, cultures expressing any of these three miRNAs show significantly reduced levels of phosphorylated AKT and FoxO3a. These results led us to suggest that these three miRNAs, miR-470, miR-669b, and miR-681, are each functionally capable of repressing the expression of IGF1R as well as AKT, inducing lower FoxO3a phosphorylation status, thus dampening the IGF1 pathway by posttranscriptional control in the brain of long-lived mouse mutants.

Results

Identification of lead miRNAs in Ames dwarf mouse brain

The comparative expression of the miRNAs of interest was screened by MM chips in brain samples of Ames dwarf mice and their wild-type counterparts of various age-groups: for dwarf mice, 2, 24, and 33 months; and for their wild-type littermate controls, 2 and 24 months. The median survival life span for control mice is approximately 28 months, and that for the Ames dwarf, approximately 38 months, hence selecting 24 and 33 months, respectively, as old age for the former and old-old age for the latter to avoid end of life span pathological complications. We have identified a list of most significantly up- and down-regulated miRNAs in dwarf mouse brain, between young and old age-groups (Table S1A,B, Supporting Information). From the results of MM Chip screening, we selected five lead up-regulated and two down-regulated miRNAs for further independent validation by primer-specific multiplex quantitative PCR assays, with the same samples used for the assays. All five up-regulated miRNAs, miR-470, miR-681, miR-669b, let-7g, and miR-501-5p, potentially target IGF1R of the somatotrophic axis. qPCR results validated the significant increase in three of them among various age-groups, miR-470, miR-681, and miR-669b, in all age-groups comparing mutant and wild-type controls (Fig. 1A).

The fold changes of the two lead down-regulated miRNAs, miR-96 and miR-709, were also validated by qPCR, as shown in Fig. 1B. However,

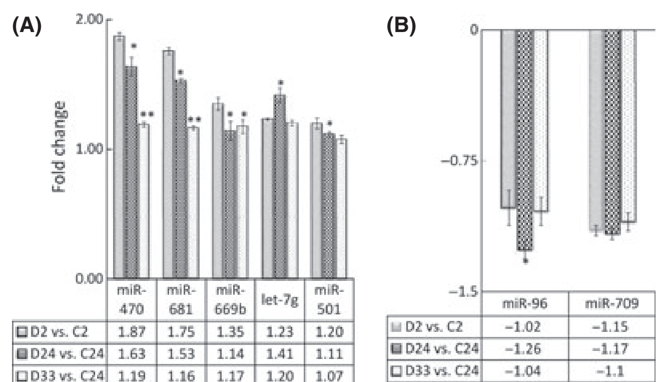

Fig. 1 Expression of key miRNAs in Ames dwarf and littermate wild-type mouse brain. A graphical representation of expression levels using qualitative PCR of key miRNAs identified by miRNA microarray (MM) chips and respective fold change values within the same age-groups among Ames dwarf and littermate controls. D2, D24, D33, C2, and C24: D represents dwarf and C represents littermate WT control mice aged 2, 24, and 33 months, respectively. (A) Shows key up-regulated miRNAs identified by MM chips, and (B) shows key down-regulated miRNAs. (*$P < 0.01$, **$P < 0.0001$; all histograms represent average ± SD; $n = 3$, three samples from each age-group of each genotype).

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these two miRNAs are also down-regulated in liver, when tissue specimens from the same age-groups are used for comparison. Both miRNAs have targets in the families of anti-apoptosis genes; their down-regulation in liver during aging has been described in detail in our previous papers, including the old dwarf mouse liver study (Maes *et al.*, 2008; Bates *et al.*, 2010). Their down-regulated expression in brain here suggests that these two miRNAs may function in a general, systemic way. Because they have no candidate targets in the IGF1 signaling axis, we have not yet studied these two miRNAs further.

MicroRNA over-expression plasmid transfection assays with the five up-regulated miRNAs show the repression of IGF1R protein expression most significantly with miR-470, miR-669b, and miR-681 with > 30% decrease, while let-7g and miR-501-5p show approximately 10% repression (Fig. S1 and Table S2, Supporting Information). Although let-7g and miR-501-5p both have potential binding sites in the 3′UTR of IGF1R according to bioinformatic data mining (data not shown), transfection assays show their functional role to be less significant than their sister miRNAs, miR-470, miR-669b, and miR-681. Similarly, except for miR-501-5p, the other four miRNAs all have binding sites on IGF1′s 3′UTR; the repression level of this gene is either none at all or < 15%. Also, only miR-470 and miR-501-5p have binding sites on PI3 kinase′s 3′UTR; the repression level of this gene is 20% or less, similar to that of let-7g. Taken together, these transfection assays led us to focus our investigation on IGF1R and the three miRNAs showing the most significant target repression, miR-470, miR-681, and miR-669b. Results shown later describe our focus on the selected three miRNAs and their shared target, IGF1R, in the IGF1 axis in long-lived mouse brain, compared with wild-type controls, as well as their direct impact on IGF signaling.

In situ detection of miRNAs in Ames dwarf mouse brain

In situ hybridization (ISH) of miR-470, miR-669b, and miR-681 shows their expression to be prominently localized in the hippocampus at various ages in Ames dwarf mice brain, using locked nucleic acid (LNA) probes, as shown in Fig. 2A,B, and C, respectively. The densitometry values from the acquired images were normalized with regard to values from a hybridization control, where a scrambled LNA probe was used. Similarly, increased LNA probe staining is also seen in the cortex of the same brain specimens. The intense localization in the hippocampus shows that this region, key to cognitive capability, indeed contains more of these three up-regulated miRNAs. These ISH data not only validate the expression profiles indicated by MM chips and qPCR but also provide further individual neuronal localization of miRNA expression, as well as their topographic profile in intact brain (Fig. 2).

Immunoblotting of key members of the GH/IGF1 axis

The putative inverse relationship between the three lead miRNAs and their candidate targets of the IGF1 signaling pathway was evaluated in the same tissue specimens used for the aforementioned studies, by immunoblotting to determine their levels of expression. We examined not only key members such as IGF1, IGF1R, PI3 kinase, AKT, and FoxO3a but also the phosphorylated forms of the last two members, because reduced PI3 kinase activity, because of either its own down-regulation or upstream decreased expression of IGF1 and/or IGF1R, results in down-regulation of phosphorylated AKT, rendering it unable to phosphorylate FoxO3a and allowing the latter to remain functional and unphosphorylated. In our Western blotting, shown in Fig. 3, the first three members,

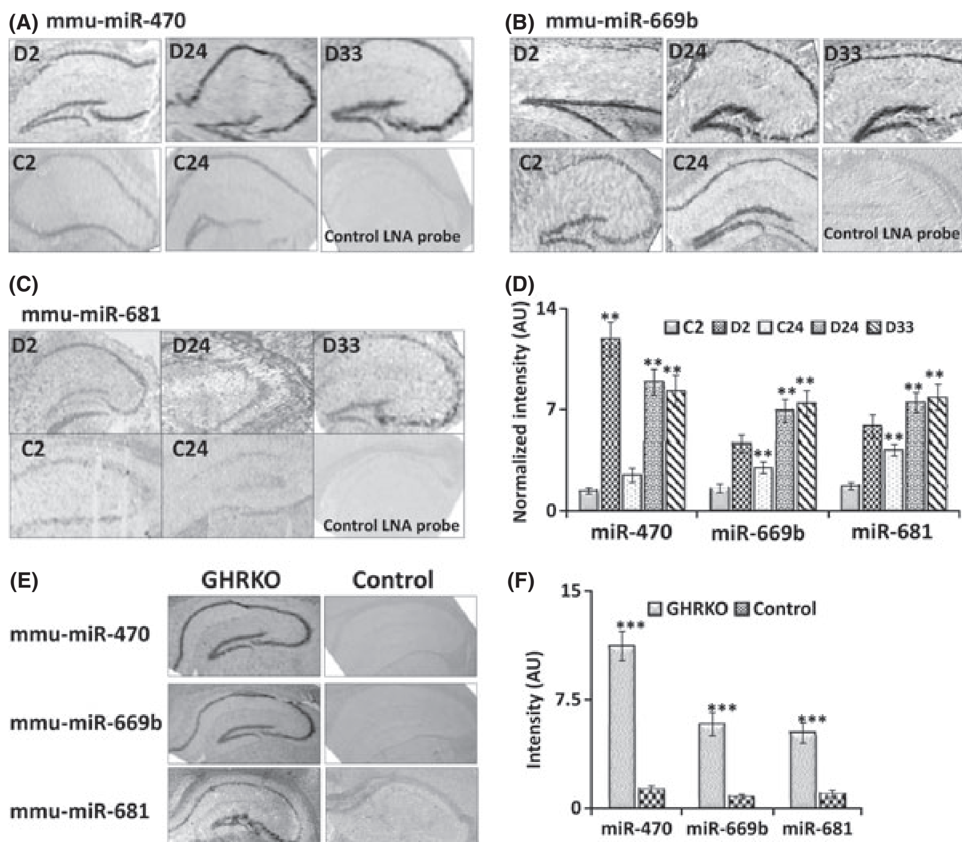

Fig. 2 *In situ* detection of lead miRNAs in Ames dwarf and growth hormone receptor gene–disrupted or knockout (GHRKO) mouse brains. *In situ* hybridization (ISH) detection of miRNAs (miR-470, miR-669b, and miR-681) in brain tissues from Ames dwarf mice (D) at 2, 24, and 33 months, depicted as D2, D24, and D33, respectively, and littermate controls (C) at 2 and 24 months, labeled as C2 and C24 age-groups (A, B, C). (D) Increased hybridization signal of these miRNAs in Ames dwarf mice across all age-groups, as compared to WT littermates, may be observed. (**P* < 0.01, ***P* < 0.0001; all histograms represent average ± SD; *n* = 3, three samples from each age-group of each genotype.) (E) *In situ* hybridization (ISH) detection of these three miRNAs in brain tissues from 2-month-old GHRKO mice and littermate controls. (F) A graphical representation of densitometric analysis of their expression in cortex and hippocampus is shown. (**P* < 0.01, ***P* < 0.0001; all histograms represent average ± SD; *n* = 3, three samples from each age-group of each genotype).

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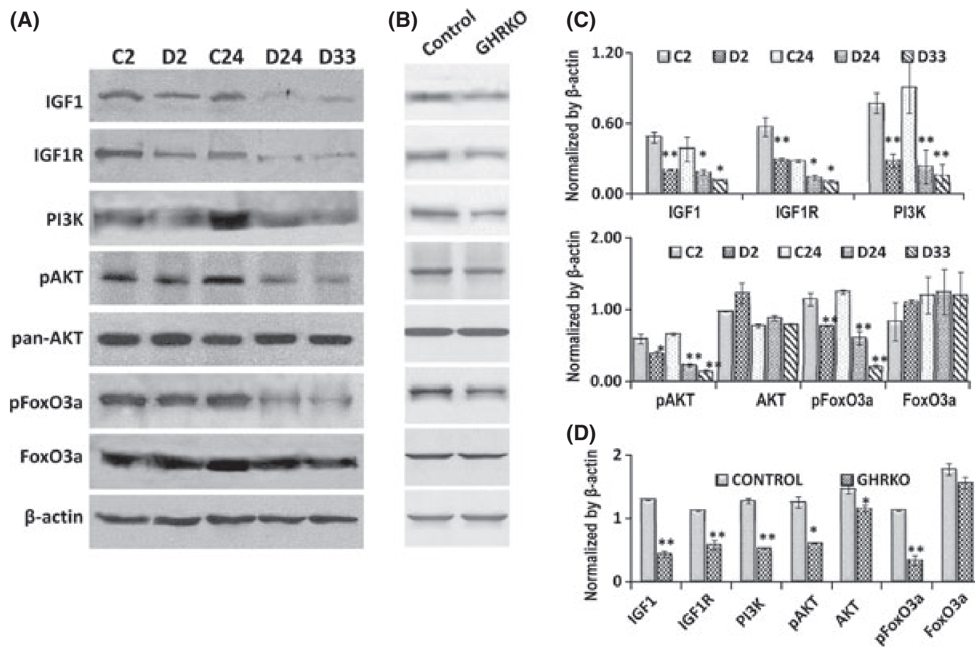

Fig. 3 Altered growth hormone signaling in Ames dwarf and growth hormone receptor gene–disrupted or knockout (GHRKO) mouse brains. In Ames dwarf mouse brain tissues (A), age-dependent decreased levels of IGF1 (14 kDa), IGF1R (95 kDa beta subunit), PI3K (85 kDa), pAKT (T308), and pFoxO3a (S253) are observed, compared to littermate WT control mice, but not in total pan-AKT (56 kDa) and FoxO3a (detected as a 100 kDa band). Similar decrease in these proteins in 2-month-old GHRKO mouse brain tissues is observed compared to littermate WT controls (B), while total pan-AKT and total FoxO3a are not significantly changed. (C) A graphical representation of densitometric analysis of Western blots of Ames dwarf mouse brain. (*$P < 0.01$, **$P < 0.0001$; all histograms represent average ± SD; $n = 3$, three samples from each age-group of each genotype.) The age-groups used are Ames dwarf at 2, 24, and 33 months, with D2, D24, and D33 labels, and the wild-type control at 2 and 24 months, represented as C2 and C24, respectively. (D) A graphical representation of densitometric analysis of Western blots of GHRKO mouse brain. (*$P < 0.01$, **$P < 0.0001$; all histograms represent average ± SD; $n = 3$, three samples from each age-group of each genotype).

IGF1 (14 kDa), IGF1R (represented by the 95 kDa beta subunit, the cytoplasmic domain), and PI3K (85 kDa), all show reduced band intensities when equal amounts of protein are loaded (Fig. 3A); this was further verified by densitometric measurements of three repeats with three different mouse brains, after standardization with the β-actin (42 kDa) band (Fig. 3C). Interestingly, the total pool levels of AKT (pan-AKT, 56 kDa, including AKT1, AKT2, and AKT3) detected by the pan-AKT antibody and total FoxO3a do not show significant differences among all five groups' comparison, but phosphorylated AKT with the phospho-threonine at amino acid residue 308 (T308) and phosphorylated FoxO3a with the phospho-serine at amino acid residue 253 (S253) are significantly reduced in abundance compared to wild-type (WT) littermates (Fig. 3A,C). As threonine 308 of AKT is phosphorylated by phosphoinositide-dependent protein kinase 1 (PDK1), the decrease in pAKT (T308) indicates that a lower level of PI3K is responsible for less AKT phosphorylation. Less activation of AKT, possibly AKT2 (Cantley, 2002; Vivanco & Sawyers, 2002), leads to less phosphorylation of FoxO3a at Ser253, making more FoxO3a translocate into the nucleus to activate its target genes, such as antioxidant genes. Taken together, these results show that indeed three members of the IGF1 cascade, IGF1, IGF1R, and PI3 kinase, are all down-regulated; AKT and FoxO3a, however, are down-regulated only in their phosphorylated forms, pAKT (T308) and pFoxO3a (S253).

Validation of lead up-regulated miRNAs of Ames dwarf mouse brain in GHRKO mutant

Based on the expression of the key up-regulated miRNAs in Ames dwarf mice as early as 2 months old, expression of miR-470, miR-669b, and miR-681 was investigated in GHRKO mouse brains at the same age. Elevated expression of these miRNAs was observed in GHRKO mice brain samples, compared to littermate WT controls (Fig. S2A, Supporting Information). Fold change values for these miRNAs from qPCR indicate their increased expression in comparison with both long-lived mouse models in (B) of Fig. S2 (Supporting Information) and in Table S2 (Supporting Information).

Expansion of ISH and immunoblotting studies in GHRKO mouse brain

Further *in situ* hybridization study with LNA probes for the three miRNAs shows prominent horseshoe localization in the hippocampus of 2-month-old GHRKO mouse brain, whereas wild-type controls display only weak staining intensity. The densitometry values from the acquired images were normalized with the values from a hybridization control where a scrambled LNA probe was used. Statistical analysis of expression of our three miRNAs of interest shows that quantitative differences between GHRKO mouse brain and wild-type controls are even more significant than those obtained by qPCR assays (Fig. 2E,F).

The inverse relationship between the three miRNAs and their candidate targets of the IGF1 axis was examined in the same tissue specimens used for the above qPCR and ISH assays. Protein profiling of 2-month-old GHRKO samples compared with WT littermates also yielded profiles similar to those in Ames dwarf specimens of the same age, with reduced expression of key IGF1 axis genes, as shown in Fig. 3B. The densitometry values from the acquired images of the blots were normalized with the values of β-actin, used as internal control, and plotted as histograms

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(Fig. 3D). These quantitations from three mouse brains show that IGF1, IGF1R, and PI3 kinase all exhibit reduced levels in GHRKO mouse brain, while a lesser reduction in the total pool for AKT and FoxO3a indicates that the expression of these genes is unchanged. For these two proteins, the significant changes are in the phosphorylated fractions, pAKT (T308) and pFoXO3a (S253), reduced to at most the same levels as IGF1, IGF1R, and PI3 kinase.

Functional determination of the lead miRNAs and their impact on their targets

To evaluate the repression of GH/IGF1 axis genes by the three lead up-regulated miRNAs identified by array screening and validated by qPCR as well as *in situ* hybridization, we over-expressed miR-470, miR-669b, and miR-681 in IGF1-responsive model cell cultures of human fibroblasts (WI-38) at early population doubling levels. Specifically, we examined the effect of these miRNAs on IGF1-induced signaling. Cultures transduced with lentivirus over-expressing miR-470, miR-669b, and miR-681 were serum-starved and subsequently stimulated by a physiologic level of IGF1, 100 ng mL^{-1}. Transduced cultures not expressing any of these three miRNAs, as well as untransduced WI-38 cultures, were used as controls, labeled 'vector alone' and 'uninfected', respectively, in the Figures. Optimal IGF1-stimulated signaling was achieved at 90 min, based on the level of pAKT (T308), although pFoxO3a (S253) peaked at 30 min after IGF1 stimulation (Fig. 4A). This observation recapitulates reports of declining pFoxO3a (S253) even though pAKT (T308) increases in rat granulosa cells upon IGF1 stimulation (Richards *et al.*, 2002). Because AKT is upstream of FoxO3a phosphorylation, we selected 90 min as a time point for experiments with > 90% transduced expression (Fig. 4B, Fig. S3, Supporting Information). Reducing IGF1R (represented by the 95 kDa beta subunit, the cytoplasmic domain of this protein) and AKT by these miRNAs exerts a downstream chain effect involving FoxO3a (Fig. 4B). As shown in Fig. 4B, quantitation of three repeated transduction experiments shows the reduction of pAKT (T308), as well as pFoxO3a (S253), with significant changes from either miR-470, miR-669b, or miR-681 transduction. In particular, cultures transduced for miR-681 expression show very low levels of total AKT, with corresponding low levels of pAKT (T308). All densitometry values from the acquired images of the blots were normalized with β-actin values, used as internal control, and plotted in Fig. 4B. The reduction of IGF1R by all three miRNAs led us to focus our reporter assays on this gene's 3′UTR in our co-transfection studies, as described later.

For the co-transfection assays, we constructed a red fluorescence reporter construct with full-length 3′UTR of IGF1R, following the protocols described in Methods. To understand how elevated expressions of the three miRNAs affect GH/IGF1 axis signaling, we conducted transient co-transfection experiments, with miR-470, miR-681, or miR-669b tagged with green fluorescence reporter constructs. At 72 h posttransfection, cultures were scored for cells exhibiting strong green fluorescence, corresponding to the miRNA constructs, and fading red fluorescence of the reporter expression of 3′UTR constructs, compared to co-transfection experiments where control vector carrying a scrambled sequence was used. Cells carrying any of the specific miRNAs or the

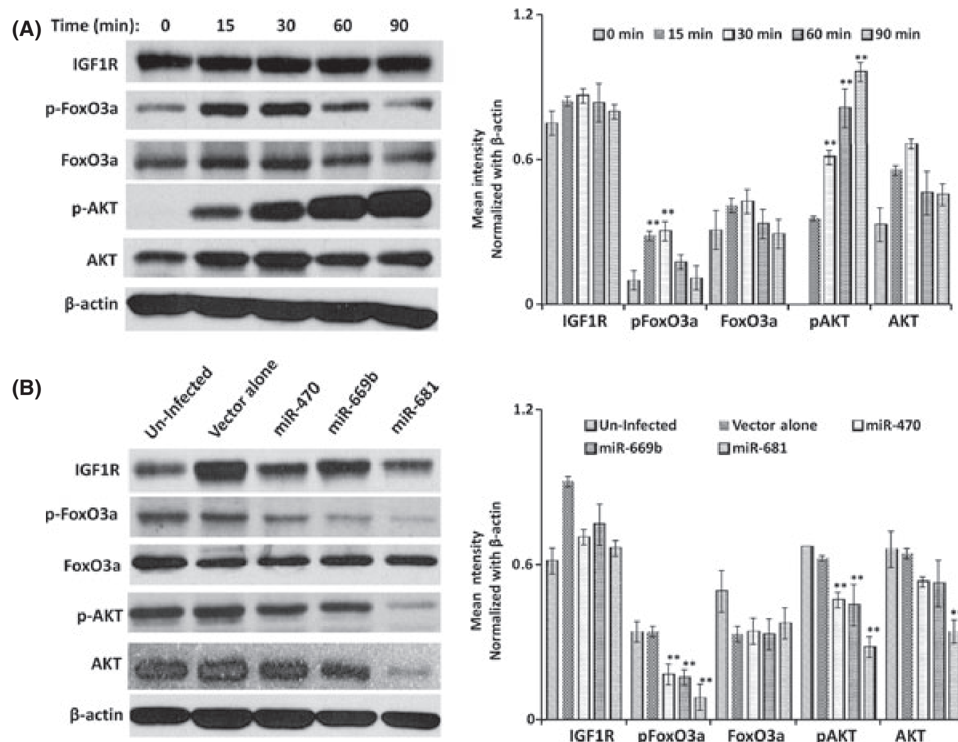

Fig. 4 MiRNA-dependent altered expression of key genes of growth hormone (GH) signaling. (A) Western blot analysis of total cell proteins at different time points after serum-deprived WI-38 cells are stimulated by IGF1. As a result of IGF1 stimulation, maximum phosphorylated Akt is seen at 90 min. Graphical representation of densitometric data showing the effect of IGF1 signaling at different time points in the form of a histogram is shown with normalized values. β-Actin was used as an internal control. (B) Western blot analysis of miRNA (miR-470, miR-669b, and miR-681) suppression of endogenous expression of GH/IGF axis genes in serum-starved and IGF1-stimulated WI-38 cells. Significant repression of IGF1R (represented by the 95 kDa beta subunit), pAKT (T308), and pFoxO3a (S253) by three key miRNAs (miR-470, miR-669b, and miR-681) is observed. No significant change in total AKt expression was observed except in miR-681-transduced cells. No significant change in total FoxO3a expression was observed. Graphical representation of densitometric data in the form of a histogram is shown with normalized values. β-Actin was used as an internal control. (*$P < 0.01$, **$P < 0.0001$; all histograms represent average ± SD; $n = 3$, three samples from each of three experiments).

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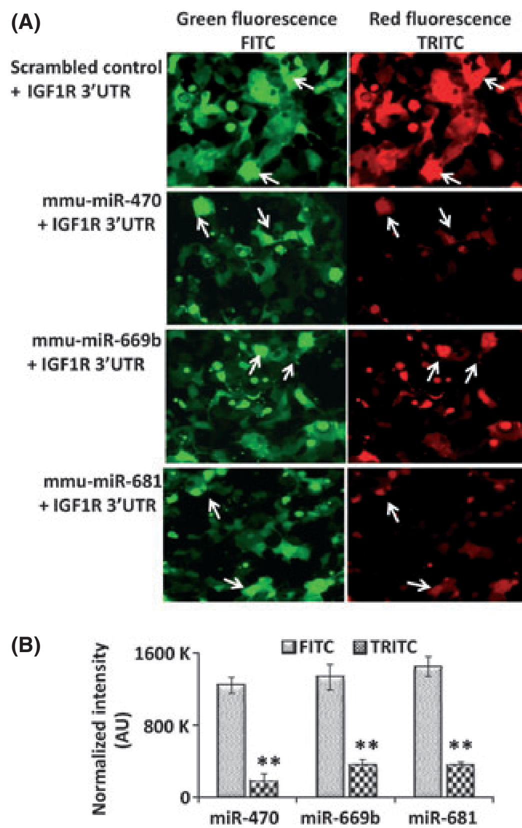

Fig. 5 MiRNA-induced repression of IGF1R 3′UTR in HEK-293 cells. (A) HEK-293 cells co-transfected with one of three key mouse miRNAs (miR-470, miR-669b, or miR-681) and IGF1R 3′UTR reporter, showing that the 3′UTR of IGF1R is repressed by the transfected miRNAs, but not by scrambled control and IGF1R 3′UTR-co-transfected cells. This indicates that these miRNAs suppress the target (red fluorescence) protein through the 3′UTR of IGF1R. This effect is absent when a plasmid carrying a scrambled sequence is used (indicated with arrows). (B) A graphical representation of densitometric analysis of color intensity is shown. (**P* < 0.01, ***P* < 0.0001; all histograms represent average ± SD; *n* = 3, three samples from each of three experiments).

IGF1R 3′UTR construct show either green with total absence of red fluorescence, or vice versa (Fig. 5A). Image analysis of the intensities between miRNA expression plasmids and reporter constructs was performed by scoring cells ($n = 1000$) showing green fluorescence with reduced red color expression. Our cumulative densitometry measurements were taken by image intensity analysis of cells ($n = 100$) from different fields ($n = 10$) from three different experiments; all values were then statistically analyzed for levels of significance (Fig. 5B). In all determinations, control plasmids carried a scrambled sequence; thus, equal intensities of green and red fluorescence served as controls. As shown, repression of IGF1R 3′UTR reporter intensity is significant in miRNA over-expression plasmid co-transfected cells, with threefold reduction, but not in controls. A combinatory transfection approach to evaluate a putative additive effect of two or three miRNAs did not result in significantly increased repression compared with single miRNA transfections (Fig. S4, Supporting Information).

Discussion

Our results from array screening, followed by qPCR studies and further individual cell localization by *in situ* hybridization assays, have unveiled the increased expression of three lead miRNAs, miR-470, miR-681, and miR-669b in long-lived mutant Ames dwarf mice. Their expressions are in inverse relationship with those of three key genes of the IGF1 signaling pathway, IGF1, IGF1R, and PI3K, in brain tissues, across all age-groups of Ames dwarf mice. Total AKT and FoxO3a, downstream from these three genes, do not share this inverse relationship, but their phosphorylated forms do. Similar inverse relationships between the three miRNAs and IGF1, IGF1R, and PI3K, along with phosphorylated AKT and phosphorylated FoxO3a, are also observed in young GHRKO mouse brain, but not in their wild-type littermates. Functional studies show that the main target for all three miRNAs is IGF1R, which exhibits the greatest reduction by the three miRNAs in transiently transfected cells, but not by two other lead miRNAs, let-7g and miR-501-5p. Cultures with transduced expression of these noncoding RNAs do not respond to IGF1-stimulated induced expression of phosphorylated AKT and FoxO3a. Interestingly, in cultures with transduced expression of miR-681, the total level of AKT is significantly reduced as well; thus, low levels of its phosphorylated isoform may be due to the combined effect of this gene's repression as well as that of IGF1R. Co-transfection with miR-470, miR-681, or -669b and the IGF1R 3′UTR construct shows that reporter expression is indeed repressed. Therefore, the three miRNAs, miR-470, miR-681, and miR-669b, share the same capability to repress the key protein, IGF1R; this is then the pivotal kernel of how GH signaling deficiency acts to reduce IGF1 signaling activity.

The fact that all three miRNAs, miR-470, miR-669b, and miR-681, are capable of repressing IGF1R of the GH signaling pathway may support the notion of an additive effect of the three working together for increased gain of suppression of GH signaling. This proves not to be the case, because our combinatory transfection approach with two or three miRNAs did not result in significantly increased inhibition beyond single miRNA transfection (Fig. S2, Supporting Information). This suggests that the three miRNAs functionally repress IGF1R independently, because their binding sites are distinct domains, with no overlap. Moreover, the 3′UTR of IGF1R is extremely long; we had to clone it by a unique recombineering approach (Liu *et al.*, 2003) to obtain the full-length clone for the repression study. The tertiary structure of IGF1R messages may give rise to a specific stoichiometric configuration with limited spatial exposure for miRNA binding to distinct binding sites. Thus, the three miRNAs sharing the same target, IGF1R, may simply reflect Nature's design increasing possible binding opportunities, rather than a synergistic additive effect among the three. In fact, this seems to be the case, because there are 14 possible sites in the IGF1R 3′UTR for miR-470 binding; this fact indeed results in the greatest repression, as seen in Fig. S2 (Supporting Information). Finally, until live image analysis of specific miRNAs binding to their targets is feasible, we are limited in our data interpretation of the temporal factors in miRNAs binding to specific targets.

Taken together, our results here indicate that (i) similar inverse relationship patterns are shared between Ames dwarf and GHRKO mouse brain at 2 months of age, between the three key miRNAs, miR-470, miR-669b, and miR-681, and key members of the IGF1 axis and (ii) the common pathway regulating the loss of function of IGF1 signaling may be due to GH signaling deficiency, as the GHRKO mice do not have the complication of the loss of two other hormones, prolactin and thyroid-stimulating hormone, seen in Ames dwarf mice. There are other long-lived mutant strains with similar GH signaling deficiency, for example, Snell dwarf mice, a closely related mutant to Ames dwarf mice with the *pit-1* gene knockdown, exhibit similar longevity. Another example of life span extension via loss of IGF1 function is heterozygous IGF1R knockout mice, whose homozygous littermates exhibit high mortality because of severe growth retardation (Flurkey *et al.*, 2001). With the lead uncovered in this report, we shall be able to address the question of whether the same trio

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of miRNAs, perhaps along with other key miRNAs, also regulates post-transcriptional repression of the IGF1 signaling axis in some of these other model systems.

The homolog of IGF1R in *C. elegans* is *daf-2*, one of the first few genes discovered to be influential in extending the worms' life span via genetic mutation (Rajah *et al.*, 1997). From worm to mouse, the IGF1 receptor is present in both the central and peripheral nervous systems, accounting for diverse molecular mechanisms underlying various neuronal functions (Rajah *et al.*, 1997). Many cell types are known to produce IGF1; in brain, the source is mainly hippocampal cells and related vasculature (Yamamoto & Murphy, 1995; Lopez-Fernandez *et al.*, 1996). Functionally, IGF1 supports the development of somatic tissues, but is not necessary for survival. For example, homozygous knockout mice such as $IGF1^{-/-}$ or $IGF2^{-/-}$ mutants are viable, although approximately 40% smaller than wild-type littermates (Liu *et al.*, 1993). In contrast, IGF1R is necessary for survival in mice; homozygous $IGF1R^{-/-}$ mice die at birth and are around 55% smaller than littermates (Liu *et al.*, 1993). Heterozygous $IGF1R^{+/-}$ not only survive to adulthood, but females exhibit extended life span. On the other hand, hyperactivation of the IGF1R signaling pathway in $p44^{+/+}$ transgenic mice leads to accelerated aging and shortening of the maximum life span (Maier *et al.*, 2004). Taken together, IGF1R expression levels may be a key to longevity; its absence results in lethality, reduced levels ensure extended life span, and overactivation results in accelerated aging and shorter life span.

The relationship of IGF1 signaling in the central nervous system to cognitive function is complex and incompletely understood. Although neurostimulatory and neuroprotective effects of IGF1 are very well documented, profound suppression of circulating IGF1 levels in GH-resistant and GH-deficient mouse mutants does not lead to cognitive deficits. Moreover, these mutants are significantly protected from age-related decline in learning and memory (Kinney *et al.*, 2001a,b). Maintenance of cognitive function in these long-lived mutants is associated with normal or increased expression of IGF1 in the brain, specifically in the hippocampus (Lupu *et al.*, 2001; Sun *et al.*, 2005a), and increased neurogenesis in the dentate gyrus (Sun *et al.*, 2005b). The apparent discrepancies between some of these reports and the present findings are likely related to methodological differences, including measurements of gene expression in the whole brain vs. dissected hippocampus. More importantly, the regulation of IGF1 receptors may be fundamentally different from the changes in expression of IGF1. Recently, Cohen *et al.* (2009) reported that heterozygous deletion of IGF1R gene in Alzheimer's model mice protects them from behavioral impairment, neuroinflammation, and neuronal loss. The present findings concerning suppression of IGF1R in the brain of long-lived mutants complement these results.

Although other miRNAs exhibit fold changes greater than 25%, that is, miR-292-3p, 28%; miR-488, 33%; miR-717, 27%; miR-705, 79%; miR-98, 42%; miR-494, 33%, etc., we selected three miRNAs, miR-470, miR-681, and miR-669b for our focus in this study because all three: (i) target IGF1R and (ii) exhibit up-regulation with age, while the other six do not. Based on our previous observation in caloric restriction and rat liver papers, several miRNAs may regulate a common signaling pathway (Khanna *et al.*, 2011; Li *et al.*, 2011b). Reduced miR-34a, miR-30e, and miR-181a-1* expression together may contribute the gain of neuronal survival in calorie-restricted mice (Khanna *et al.*, 2011). Similarly, the increase in miR-34a, miR-93, etc. may functionally impact the signaling pathway of oxidative defense in old rat liver (Li *et al.*, 2011b). Therefore, our selection of these three miRNAs as our focus is based on their shared functional impact on the same gene, IGF1R, rather than the conventional approach of selecting those with the greatest changes. Likewise, even

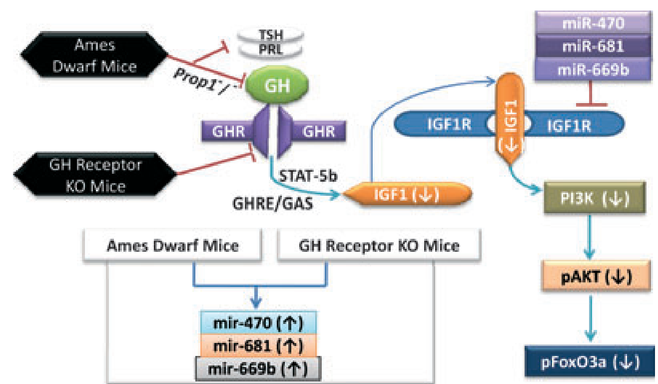

Fig. 6 MiRNA-mediated altered overlapping somatotropic signaling in long-lived mice. This schematic diagram shows the common miRNA-based regulation in both long-lived mouse models (Ames dwarf and growth hormone receptor gene–disrupted or knockout mice). It depicts the role of three key miRNAs (miR-470, miR-669b, and miR-681) targeting a key gene, IGF1R, of the growth hormone (GH)/IGF1 axis, and altering GH signaling, thus contributing to longevity.

though repression of AKT is observed in miR-681-transduced human fibroblast cultures, it is not shared by the other two miRNAs, miR-470 and miR-669b. The particular focus on the functional relationship between miR-681 and AKT repression shall be pursued in future experiments, testing whether this single miRNA is dominant among the three miRNAs discussed here and whether it exerts an additional effect on controlling the IGF signaling pathway.

Our present results suggest the schematic diagram depicted in Fig. 6, showing the miRNA-based molecular pathway in GH-deficient signaling seen in both Ames dwarf and GHRKO mouse brain. Our results suggest for the first time a mechanism for the direct suppression of IGF1R by three lead miRNAs, miR-470, miR-669b, and miR-681 to regulate a signaling cascade in the loss-of-function operating modality. It remains to be elucidated in future work why and how these three miRNAs are up-regulated in the GH-signaling-deficient environment in these two mutant mice. Discovering the transcription factors activating these miRNAs in GH-deficient long-lived mice may provide future leads to express them in wild-type mice, affording a gain of cognitive robustness and health span like that seen in the long-lived mutants, the ultimate dream of longevity study.

Experimental procedures

Animals

Ames dwarf mice and their normal (N) littermates were produced in Dr. Bartke's laboratory at the Southern Illinois University School of Medicine. GHRKO mice and normal littermate controls were produced in a closed colony derived from animals provided by Dr. J. Kopchick and maintained at Southern Illinois University. All animals were housed under controlled temperature and light conditions (20–23°C, 12-h light and 12-h dark cycle) and provided *ad libitum* nutritionally balanced diet (Rodent Laboratory Chow 5001: 23.4% protein, 4.5% fat, 5.8% crude fiber; LabDiet PMI Feeds, Inc., St Louis, MO, USA). All procedures were approved by the Laboratory Animal Care and Use Committee at Southern Illinois University School of Medicine. Total brain tissue samples were dissected out from Ames dwarf, GHRKO mice, and respective normal wild-type littermates of various age-groups. Tissue samples were then shipped to University of Louisville on dry ice. All animal tissue-related processing and experiments were conducted in accordance with approved institutional (University of Louisville) biosafety board protocol #05-001.

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Table 1 Properties and sources of the antibodies used in this study

Name	Vendor catalog #	Antigenic specificity
Rabbit anti-IGF polyclonal antibody	ab9572	Human & mouse IGF1 of 14 kDa
Rabbit anti-pan-AKT polyclonal antibody	ab8805	Human & mouse AKT of 56 kDa
Rabbit anti-pAKT polyclonal antibody	ab38449	Human AKT with phosphor-threonine at amino acid 308 site
Rabbit anti-FoxO3a polyclonal antibody	ab47409	Human FoxO3a of 70 kDa
Rabbit anti-pFoxO3a polyclonal antibody	ab47285	Human FoxO3a with phosphor-serine at amino acid 253 site
Rabbit anti-tubulin polyclonal antibody	ab4074	Human & mouse tubulin of 50 kDa
Mouse anti-β actin monoclonal antibody	ab8226	Human & mouse β-actin of 42 kDa
Mouse anti-PI3k monoclonal antibody	ab86714	Human & mouse PI3 Kinase of 85 kDa
Rabbit anti-IGF1R polyclonal antibody	NB100-81979	Human & mouse β-subunit of 95 kDa

ab stands for Abcam Inc. Cambridge, MA, USA.
NB stands for Novus Biologicals, Littleton, CO, USA.

Antibodies and Western blotting

Antibodies used are included in Table 1 to list their vendor sources and antigenic specificity. Brain tissues from Ames dwarf, GHRKO mice, and respective wild-type littermate controls (2, 24, and 33 months for Ames dwarf mice and 2 months for GHRKO mice, n = 3 each) were homogenized as described previously (Bates *et al.*, 2010). Protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA, USA). Following electrophoresis and transfer onto membranes, the membranes were incubated overnight at 4°C with primary antibodies. Blots were developed and analyzed as described in our previous publication (Bates *et al.*, 2010). Antibodies used are included in Table 1 to list their vendor sources and antigenic specificity. Among these antibodies, the most important ones are those to IGF1R, AKT, and FoXO3a. The antibody to IGF1R detects the beta subunit of this protein, which is the cytoplasmic domain, differing from the α-subunit located outside the plasma membrane for binding with the IGF1 ligand. The antibody (ab8805) to the total pool of AKT was produced to amino acids 466–480, a common domain of human AKT1, AKT2, and AKT3, with 100% similarity to those of rat, chicken, and mouse, and detects a 56-kDa band. Phosphorylated AKT (pAKT) was detected by an antibody (ab38449) detecting only phosphorylated AKT with its phosphor-moiety residue at threonine 308 and therefore termed here pAKT (T308). Thus, two antibodies were used to identify the total pool vs. the T308-phosphorylated form of AKT. Likewise, antibody ab47409 was used to detect the total pool of FoXO3a, while antibody ab47285 detects this protein's phosphorylated form, with its phosphor-moiety at the serine 253 amino acid residue. Phosphorylated FoXO3a detected by the latter antibody is termed throughout the text as the S253 isoform of this protein.

Procedures

A detailed experimental procedures were described in the Data S1 (Supporting Information), including (i) miRNA microarray profiling; (ii) mouse tissue samples processing; (iii) total and small RNA extractions; (iv) protein extraction; (v) qRT-PCR validation; (vi) miRNA *in situ* hybridization; (vii) construction of miRNA over-expression plasmid and IGF1R 3′-UTR reporter; (viii) cell transduction and transfection; and (ix) statistical analysis.

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Authors contributions

A.K., H.S., S.M., and E.W. contributed to the acquisition, analysis, and interpretation of the data. S.M. and E.W. did the MM chip design. R.L. designed and made all the clones and pseudolentivirus used in the study. N.L. contributed to the Ames dwarf brain *in situ* hybridization. A.B., J.J.K., and M.M. helped fine-tune the manuscript and provided animal tissue samples used in the study. J.J.K. generated the transgenic mouse line of GHRKO. A.K., R.L., and E.W. designed the study and wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1 Detailed experimental procedures were described in the supplemental text.

Fig. S1 MiRNA-dependent altered expression of key genes of GH signaling, and repression of IGF1R 3′UTR in HEK-293 cells.

Fig. S2 Expression of lead miRNAs in brain of GHRKO mice compared with control littermates, and fold change comparison between GHRKO and Ames dwarf mice at 2 months of age.

Fig. S3 Transduction of WI-38 cells with lentivirus over expressing miR-470, -669b, -681 and controls.

Fig. S4 Combinatoric miRNA-induced repression of IGF1R 3′UTR in HEK-293 cells.

Table S1 Identification of key miRNAs and their expression in Ames dwarf mice.

Table S2 Fold change values comparing expression of lead miRNAs in Ames dwarf and GHRKO mice with their respective control littermates.

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