AGE (2016) 38:239–258
DOI 10.1007/s11357-016-9943-9

ORIGINAL ARTICLE

IGF-1 deficiency in a critical period early in life influences the vascular aging phenotype in mice by altering miRNA-mediated post-transcriptional gene regulation: implications for the developmental origins of health and disease hypothesis

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Received: 23 April 2016 / Accepted: 29 July 2016 / Published online: 26 August 2016
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Abstract Epidemiological findings support the concept of Developmental Origins of Health and Disease, suggesting that early-life hormonal influences during a sensitive period of development have a fundamental impact on vascular health later in life. The endocrine changes that occur during development are highly conserved across mammalian species and include dramatic increases in circulating IGF-1 levels during adolescence. The present study was designed to characterize the effect of developmental IGF-1 deficiency on the vascular aging phenotype. To achieve that goal, early-onset endocrine IGF-1 deficiency was induced in mice by knockdown of IGF-1 in the liver using Cre-lox technology (*Igf1*$^{f/f}$ mice crossed with mice expressing albumin-driven Cre recombinase). This model exhibits low-circulating IGF-1 levels during the peripubertal phase of development, which is critical for the biology of aging. Due to the emergence of miRNAs as important regulators of the vascular aging phenotype, the effect of early-life IGF-1 deficiency on miRNA expression profile in the aorta was examined in animals at 27 months of age. We found that developmental IGF-1 deficiency elicits persisting late-life changes in miRNA expression in the vasculature, which significantly differed from

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those in mice with adult-onset IGF-1 deficiency (TBG-Cre-AAV8-mediated knockdown of IGF-1 at 5 month of age in *Igf1*$^{f/f}$ mice). Using a novel computational approach, we identified miRNA target genes that are co-expressed with IGF-1 and associate with aging and vascular pathophysiology. We found that among the predicted targets, the expression of multiple extracellular matrix-related genes, including collagen-encoding genes, were downregulated in mice with developmental IGF-1 deficiency. Collectively, IGF-1 deficiency during a critical period during early in life results in persistent changes in post-transcriptional miRNA-mediated control of genes critical targets for vascular health, which likely contribute to the deleterious late-life cardiovascular effects known to occur with developmental IGF-1 deficiency.

Keywords Insulin-like growth factor 1 · miRNA · Epigenetics · Post-transcriptional regulation · microRNA

Introduction

Epidemiological findings during the past two decades support the concept of Developmental Origins of Health and Disease, suggesting that early-life events during a sensitive period of development have a fundamental impact on the organism's later development, tissue structure and function and lifespan (Barker 2004a, b; Gillman 2005). Increasing clinical and experimental evidence, including parabiotic studies, suggest that the endocrine milieu present during development, especially when rapid physical growth occurs, induces cellular programs that affect the pathogenesis of age-related disease (Barker et al. 1989, 2005; Bateson et al. 2004; Eriksson et al. 1999, 2000, 2007; Kajantie et al. 2005; Osmond et al. 2007).

The endocrine changes that occur during development are highly conserved across mammalian species and include dramatic increases in the anabolic hormone IGF-1 during adolescence (due to a significant rise in GH secretion) (Carter et al. 2002; D'Costa et al. 1993; Deak and Sonntag 2012; Sonntag and Csiszar 2012; Sonntag et al. 2000, 1999, 2005b). Levels of circulating IGF-1 can increase several folds during this period compared to pre-adolescent levels. Yet, this increase is highly variable (Edouard et al. 2009; Sorensen et al. 2012). There is increasing experimental and clinical evidence that alterations in IGF-1 levels during development regulate multiple aspects of the aging process and affect the incidence of multiple age-related diseases (Sadagurski et al. 2015; Sonntag and Csiszar 2012). Importantly, developmental IGF-1 deficiency was suggested to extend lifespan in certain murine models of aging, including the Ames dwarf mice and Snell dwarf mice (Panici et al. 2010). The lifespan-extending effects of developmental IGF-1 deficiency have been largely attributed to its anti-cancer effects (Ikeno et al. 2003). On the basis of these observations, hypotheses were put forward proposing that developmental IGF-1 level is an evolutionarily conserved mechanism regulating the aging process (Bartke and Brown-Borg 2004).

The cardiovascular system is an especially important target organ for IGF-1 (Chisalita and Arnqvist 2004; Chisalita et al. 2009; Johansson et al. 2008; Li et al. 2007; Toth et al. 2014, 2015), and there is increasing evidence suggesting that early-life IGF-1 levels may determine cardiovascular health in later life (Sonntag et al. 2005a, 2013). Accordingly, previous studies demonstrate that rodent models with developmental IGF-1 deficiency exhibit a cardiac and/or vascular phenotype in adulthood (Csiszar et al. 2008; Helms et al. 2010; Reddy et al. 2014). For example, adult growth hormone-releasing hormone receptor null dwarf (Little) mice have significantly lower peak and mean aortic velocity and significantly higher aortic impedance than young wild-type mice (Reddy et al. 2014). Adult Ames dwarf mice exhibit cardiac and vascular mitochondrial oxidative stress (Csiszar et al. 2008), whereas adult GH/IGF-1-deficient Lewis dwarf rats exhibit impaired cardiac performance (Cittadini et al. 1997; Longobardi et al. 2000) and impaired vascular stress resistance phenotypes (Bailey-Downs et al. 2012b; Ungvari et al. 2010). However, the mechanistic role of developmental IGF-1 deficiency in regulation of the vascular aging process remains obscure.

MicroRNAs (miRNA) are short, endogenous, non-coding transcripts that regulate the expression of specific messenger RNA (mRNA) targets (Lee et al. 2014; Liu et al. 2015). There is growing evidence that miRNAs control lifespan and the pace of aging in model organisms (Boehm and Slack 2005; Grillari and Grillari-Voglauer n.d.; Ibanez-Ventoso et al. 2006) and that changes in miRNA expression profile also have a role in mammalian aging (Bates et al. n.d.; Inukai et al. 2012; Inukai and Slack 2013; Ito et al. 2010; Maes et al. 2008; Mercken et al. 2013; Smith-Vikos and Slack 2012;

Ungvari et al. 2013; Zhang et al. 2012; Zovoilis et al. 2011). Importantly, miRNAs were also reported to regulate several important aspects of endothelial biology and vascular function (Bonauer et al. 2009; Chen et al. 2015; Doebele et al. n.d.; Hergenreider et al. 2012; Kim et al. 2014; Kuehbacher et al. 2007; Leung et al. 2013; Lovren et al. 2012; O'Rourke and Olson 2011; Rotllan et al. 2013; Stellos and Dimmeler 2014; Weber et al. 2014; Zampetaki et al. 2014). Further, age-related changes in miRNA expression were shown to contribute to the development of cardiovascular aging phenotypes (Boon et al. 2013; Csiszar et al. 2014; Ungvari et al. 2013) and the pathogenesis of cardiovascular diseases (Ono et al. 2011). Expression of miRNAs in the cardiovascular system was reported to be regulated by neuroendocrine factors (Hua et al. 2012). Despite these advances, the effects of developmental IGF-1 deficiency on vascular miRNA expression profile have not been elucidated.

The present study was designed to characterize the effect of developmental IGF-1 deficiency on the vascular aging phenotype. To achieve that goal early-onset, isolated endocrine IGF-1 deficiency was induced mice by developmental knockdown of IGF-1 specifically in the liver using Cre-lox technology (*Igf1*$^{f/f}$ mice crossed with mice expressing albumin-driven Cre recombinase) (Ashpole et al. 2015). The animals were studied at an age representing ~75 % of maximal lifespan potential, which corresponds to the biological age of a ~67-year-old human. To assess vascular health, endothelium-dependent vasorelaxation and vascular ROS production were tested. Due to the emergence of miRNAs as important regulators of vascular aging phenotype (Csiszar et al. 2014; Ungvari et al. 2013), miRNA expression profile in the aorta of mice with developmental IGF-1 deficiency was tested.

Materials and methods

Developmental liver-specific knockdown of Igf1 in mice

To target IGF-1 production early in development, mice homozygous for a floxed exon 4 of the *Igf1* gene (*Igf1*$^{f/f}$; in a C57BL/6 background (Toth et al. 2014)) were crossed with mice expressing albumin-driven Cre recombinase, as previously described (Ashpole et al. 2015). The *Igf1*$^{f/f}$ mice have the entirety of exon 4 of the *Igf1* gene flanked by loxP sites, which allows for genomic excision of this exon when exposed to Cre recombinase. Transcripts of the altered *Igf1* gene yield a protein upon translation that fails to bind the IGF receptor. The albumin gene is induced within the liver between post-natal day 10 and 15, thereby decreasing effective IGF-1 production early after birth. Knockdown of IGF-1 was verified by measuring circulating levels of IGF-1 at 2, 12, and at 27 months of age as reported (Ashpole et al. 2015). Mice were used for experimentation at 27 months of age. Wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and utilized as reference controls at 5 and 27 months of age.

As an additional control group, aortas isolated from mice with adult-onset IGF-1 deficiency were also analyzed (Fig.1a). Adult-onset circulating IGF-1 deficiency was induced in *Igf1*$^{f/f}$ mice by adeno-associated virus (AAV8)-mediated expression of Cre recombinase in the liver at 5 months of age, as reported (Ashpole et al. 2015). The AAV8 vector was purchased from the University of Pennsylvania Viral Vector Core (Penn Vector Core, Philadelphia, PA, USA; http://www.med.upenn.edu/gtp/vectorcore). Although AAV8 is effective at transducing multiple tissues, the use of thyroxine binding globulin (TBG) promoter allows for the restriction of expression to hepatocytes, as described (Toth et al. 2014). At 5 months of age, *Igf1*$^{f/f}$ mice were administered approximately 1.3 × 1010 viral particles of AAV8-TBG-Cre or AAV8-TBG-eGFP via retro-orbital injection, as described (Ashpole et al. 2015; Toth et al. 2014).

Animals were housed in the Rodent Barrier Facility at the University of Oklahoma Health Sciences Center, on a 12-h light/12-h dark cycle, and given access to standard rodent chow (Purina Mills, Richmond, IN) and water ad libitum. All procedures were approved by and followed the guidelines of the Institutional Animal Care and Use Committee of OUHSC in accordance with the ARRIVE guidelines.

Measurement of circulating IGF-1 levels

Venous blood was collected from the submandibular veins of animals from each group (Medipoint, Mineola, NY). Whole blood was centrifuged at 2500×*g* for 20 min at 4 °C to collect serum, which was then stored at −80 °C. Measurement of serum IGF-1 (Franco et al. 2014; Hill et al. 2015; Rojanathammanee et al. 2014)

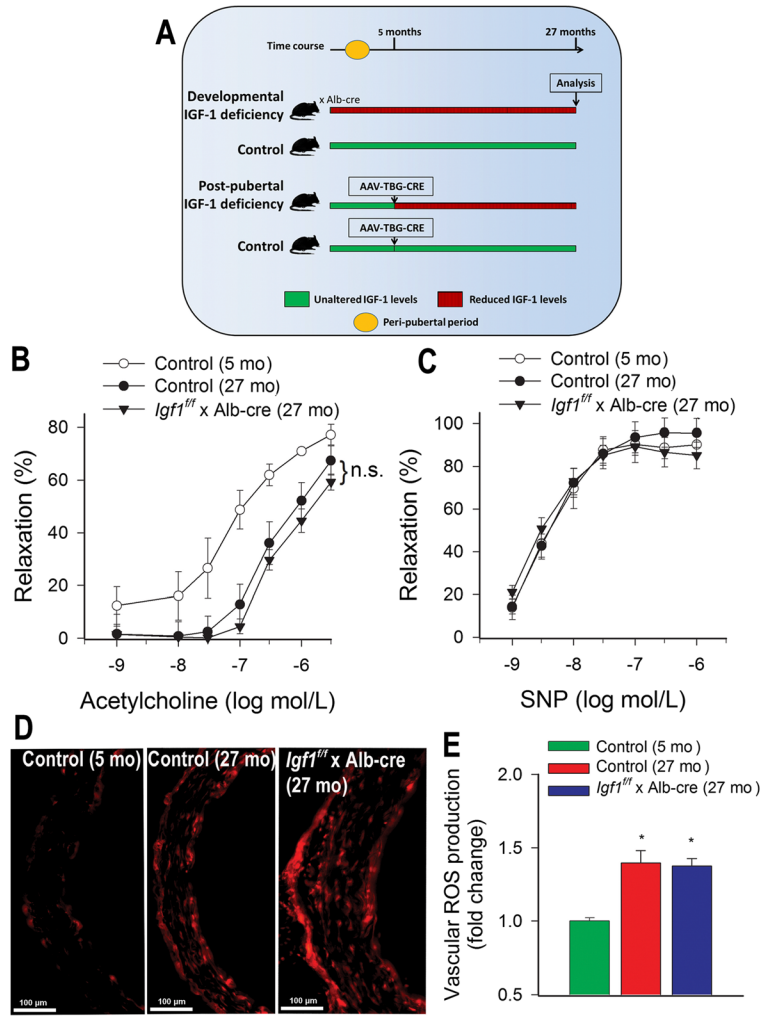


Fig. 1 Age-related endothelial dysfunction and increased oxidative stress in aortas from mice with developmental IGF-1 deficiency. **a** Experimental scheme. As a model of developmental IGF-1 deficiency *Igf1*$^{f/f}$ mice crossed with mice expressing albumin-driven Cre recombinase (*Igf1*$^{f/f}$ *x* Alb-cre*)* were used. As a model of adult-onset, post-pubertal IGF-1 deficiency *Igf1*$^{f/f}$ mice were injected with TBG-iCre-AAV8 at 5 months of age. Mice were analyzed at 27 month of age. **b**–**c** Relaxations in ring preparations of aortas of aged (27 months old) *Igf1*$^{f/f}$ *x* Alb-cre mice and aged (27 months old) and young (5 months old) control mice in response to administration of increasing concentrations of acetylcholine (**b**) and the NO donor SNP (**c**). Data are mean ± SEM (n = 6–8). **d** Representative confocal images showing ethidium fluorescence (representing increased ROS levels) in section of aortas of aged (27 months old) *Igf1*$^{f/f}$ *x* Alb-cre mice and aged (27 months old) and young (5 months old) control mice. Summary data for vascular ROS production are shown in **e**. Data are mean ± S.E.M. *$p < 0.05$ vs. young control

levels was performed by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol and are reported in ng/mL.

Assessment of vascular endothelial function

Upon euthanasia, aortas were isolated and endothelial function was assessed by measuring relaxation of aortic ring preparations to acetylcholine as previously described (Bailey-Downs et al. 2012a). Endothelial function is an important measure of vascular health (Alonso-Bouzon et al. 2014; Demirci et al. 2014; Gonzalez-Guardia et al. 2014; Grabowska et al. 2015; Heiss et al. 2015; Mourmoura et al. 2014; Walker et al. 2014). In brief, an aorta ring segment 2 mm in length was isolated from each animal and mounted on 40-µm stainless steel wires in myograph chambers (Danish Myo Technology A/S, Inc., Denmark) for measurement of isometric tension. The vessels were superfused with Krebs buffer solution (118 mM NaCl, 4.7 mM KCl,

1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 5.6 mM glucose; at 37 °C; gassed with 95 % air and 5 % CO_2). After an equilibration period of 1 h during which an optimal passive tension was applied to the rings (as determined from the vascular length-tension relationship), relaxation of pre-contracted (by 10^{-5} mol/L phenylephrine) vessels to the endothelium-dependent vasodilator acetylcholine (ACh; from 10^{-9} to 10^{-6} mol/L) and to an endothelium-independent vasodilator, the NO donor sodium nitroprusside (SNP; from 10^{-9} to 10^{-6} mol/L) was obtained.

Measurement of vascular ROS production

The oxidative fluorescent dye dihydroethidium (DHE) was used to assess vascular O_2^- production in segments of the aortas as we have previously reported (Csiszar et al. 2007; Pearson et al. 2008; Ungvari et al. 2003, 2010). In brief, freshly isolated aorta segments were incubated with DHE (3×10^{-6} mol/L; for 30 min, at 37 °C, in the dark). The vessels were then washed three times, embedded in OCT medium and cryosectioned. Fluorescent images of the aorta sections were captured using a Leica SP2 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). Average nuclear DHE fluorescence intensities were assessed using the Metamorph software (Molecular Devices LLC, Sunnyvale, CA) and values for each animal in each group were averaged as reported (Csiszar et al. 2007; Pearson et al. 2008; Ungvari et al. 2003, 2010). Unstained aortas were used for background correction.

Quantitative real-time RT-PCR

A quantitative real time RT-PCR technique was used to analyze miRNA expression profiles in the aorta of mice from each experimental group as reported (Csiszar et al. 2014). In brief, total RNA was isolated with a mirVana™ miRNA Isolation Kit (ThermoFisher Scientific) and was reverse transcribed using TaqMan® MicroRNA Reverse Transcription Kit as described previously (Bailey-Downs et al. 2012a; Csiszar et al. 2014). The expression profile of 641 unique mouse miRNAs in aortas derived from young and aged control mice and aged mice with developmental IGF-1 deficiency was analyzed using the TaqMan Array Rodent MicroRNA A + B Cards Set v3.0 (ThermoFisher Scientific). Expression of miRNAs were normalized to ΔΔCt values using the average of three replicated probes of MammU6, and the resulting expression values were then quantile normalized (Csiszar et al. 2014). Differential expression raw p values were determined using a Student's t test and corrected using Benjamini-Hochberg multiple hypothesis correction at a q-value (FDR) cutoff of 0.1.

miRNA target prediction and validations

To further understand the consequences of changes in miRNA abundance on regulating vascular aging phenotypes, we used a computational approach to predict targets of differentially expressed miRNAs. After determining miRNAs that were differentially expressed with developmental IGF-1 deficiency and age, we compiled a list of candidate target genes matching the following criteria: (1) Putative targets of miRNAs differentially expressed in aortas of $Igf1^{f/f}$xAlb-cre mice, (2) expressed in aorta and show altered expression with age in aorta, (3) co-expressed with IGF-1 across tissues and experimental conditions, and (4) associated with vascular pathophysiology in the published literature. For criterion (1), a list of miRNA-target pairs was obtained from miRBase (Kozomara and Griffiths-Jones 2014), and for each target gene in the database, the number of targeting miRNAs that were significantly up- or downregulated in aging or developmental IGF-1 deficiency was quantified and significance was assessed using the binomial test. The hypothesis tested was that the targeting miRNAs were consistent in their direction of regulation with IGF-1 deficiency or age. For criterion (2), mouse RNA microarray samples were identified in NCBI GEO as deriving from aorta using GEOmetadb (Zhu et al. 2008). Each aorta sample from GEO series accession GSE40156 was annotated with the sample age, and after quantile normalization, a log-linear model was used to quantify the rate of expression change of each gene with time. Additionally, the mean expression of each gene in aorta was approximated by converting each sample's log-expression vector to a Z-score. Genes with a mean Z-score less than 0 (indicating genes which were expressed at a lower level than the average gene) were excluded from further analysis. For criterion (3), the GAMMA algorithm (Dozmorov et al. 2011; Wren 2009) was used to quantify the correlation of each putative target mRNA with the IGF-1 transcript using the Pearson's correlation coefficient. For criterion (4), the IRIDESCENT algorithm (Wren and Garner 2004)

was used to mine the biomedical literature and quantify the degree of association between each candidate mRNA and terms relating to vascular pathophysiology (e.g., "stroke," "aneurysm," "vascular fragility," "ischemic heart disease," etc.). IRIDESCENT uses a statistical model to determine whether each gene co-occurs with a term of interest (here, vascular pathophysiology-related terms) more frequently than would be expected by chance, and quantifies this in terms of the mutual information measure. In order to retrieve the most relevant targets, we chose the top-ranked miRNA target genes predicted by these computational approaches. We next validated these predictions with quantitative real-time RT-PCR using TaqMan probes as reported (Csiszar et al. 2013; Toth et al. 2013; Tucsek et al. 2013, 2014).

Statistical analysis

Statistical analysis was carried out by one-way ANOVA followed by Tukey's post-hoc test or unpaired *t* test, as appropriate. Dose-response curves for vascular relaxations were analyzed by two-way ANOVA for repeated measures followed by Bonferroni multiple comparison test. A *p* value less than 0.05 was considered statistically significant. Data are expressed as mean ± S.E.M.

Results

Developmental liver-specific knockdown of IGF-1

Basic physiological parameters of the experimental cohorts used in the present study were similar to our previous report (Ashpole et al. 2015). Body weight was significantly decreased in the *Igf1*$^{f/f}$xAlb-cre and *Igf1*$^{f/f}$ + AAV8-TBG-Cre groups, compared to their age-matched controls (Table 1). Similarly, circulating IGF-1 levels were significantly reduced in the *Igf1*$^{f/f}$xAlb-cre and *Igf1*$^{f/f}$ + AAV8-TBG-Cre groups, compared to their respective age-matched controls (Table 1).

Endothelial dysfunction and oxidative stress

IGF-1 is known to exert multifaceted vasoprotective effects (Bailey-Downs et al. 2012a, b; Csiszar et al. 2008; Higashi et al. 2010, 2012; Sonntag et al. 2013; Sukhanov et al. 2007; Ungvari and Csiszar 2012; Ungvari et al. 2010) but the role of developmental IGF-1 deficiency in regulating vascular aging has never been investigated. We found that endothelium-dependent aorta relaxation induced by acetylcholine was significantly impaired in aged control mice as compared to young control mice (Fig. 1b). There was no significant difference between acetylcholine-induced responses in aortas of aged *Igf1*$^{f/f}$xAlb-cre mice and aged control mice (Fig.1b). We also investigated the effect of the endothelium-independent relaxing agent SNP, and we found that there was no significant difference among the groups (Fig. 1c).

Analysis of nuclear ethidium fluorescence intensities showed that aging was associated with significant increases in vascular O_2^- production in control mice (Fig. 1d–e). There was no significant difference between O_2^- production in aortas of aged *Igf1*$^{f/f}$xAlb-cre mice and aged control mice (Fig. 1d–e).

Table 1 Description of experimental animals

Group	n	Age (days)	Body weight (grams)	IGF-1 levels (ng/mL)
5-month-old control	7	n.a.	25.7 ± 1.5	320.9 ± 65.4
27-month-old control	7	823.4 ± 2.6	26.8 ± 3.3	314.0 ± 49.9
Igf1$^{f/f}$ x Alb-Cre	7	823.4 ± 1.9	23.2 ± 1.8*	46.4 ± 12.0*
Igf1$^{f/f}$ x TBG-eGFP-AAV8	7	821.9 ± 5.6	26.4 ± 1.1	310.8 ± 68.0
Igf1$^{f/f}$ x TBG-Cre-AAV8	7	820.3 ± 5.8	23.5 ± 1.3*	53.2 ± 10.4*

Average age, body weight, and circulating IGF-1 levels at the time of tissue harvest in each experimental group. The asterisk indicates a significant difference between the treatment group and its respective control group, *$p < 0.05$, mean ± S.D

n.a. data not available

Changes in vascular miRNA expression profile in mice associated with aging and with developmental IGF-1deficiency

We assessed changes in miRNA expression profile in the mouse aorta associated with aging and with developmental IGF-1 deficiency. Principal component analysis and hierarchical clustering of miRNA expression showed a clear separation between the young and aged groups. Aged control mice and aged *Igf1*$^{f/f}$xAlb-cre mice were also separated in the principal component analysis and hierarchical clustering. Figure 2a, b shows changes in miRNA expression in the mouse aorta associated with age and developmental IGF-1 deficiency, respectively. GO terms enriched among miRNAs differentially expressed with age and developmental IGF-1 deficiency are shown in Table 2 and Table 3, respectively.

To differentiate between the effects of IGF-1 deficiency during development and post-pubertal IGF-1 deficiency on vascular phenotype, miRNA expression in aortas of *Igf1*$^{f/f}$xAlb-cre mice and *Igf1*$^{f/f}$ + AAV8-TBG-Cre mice was compared. Figure 2c shows that expression of miRNAs that are differentially expressed in the aortas of *Igf1*$^{f/f}$xAlb-cre mice was not altered significantly in aortas of *Igf1*$^{f/f}$ + AAV8-TBG-Cre mice, suggesting that developmental IGF-1 status has a critical role in regulation of vascular miRNA expression. The only exception identified was miR-204 whose expression was similarly altered both in *Igf1*$^{f/f}$xAlb-cre mice and *Igf1*$^{f/f}$ + AAV8-TBG-Cre mice *Igf1*$^{f/f}$xAlb-cre mice and *Igf1*$^{f/f}$ + AAV8-TBG-Cre mice.

Changes in vascular expression of miRNA target genes

Since the discovery of miRNA regulation of genes, several studies have been focused on predicting the biologically relevant target genes for miRNAs. We have designed a novel selection strategy to predict putative biological targets of differentially expressed miRNAs as shown in Fig. 3a. The top-ranked miRNA target genes predicted by these computational approaches were validated using qPCR. We found that our method successfully predicted miRNA target genes whose aortic expression is significantly impacted by developmental IGF-1 deficiency and age (Fig. 3b). The effects of developmental IGF-1 deficiency and aging on aortic mRNA expression of predicted biological targets of differentially regulated miRNAs are shown in Fig. 3c. Comparison of aortic expression of selected miRNA targets shows that developmental IGF-1 deficiency (*Igf1*$^{f/f}$ *x* Alb-cre) and adult-onset IGF-1 deficiency (*Igf1*$^{f/f}$ + TBG-iCre-AAV8) differentially alter expression of a number of targets genes related to extracellular matrix homeostasis and maintenance of vascular structural integrity (Fig. 3d).

Discussion

The principal new finding of this study is that IGF-1 deficiency through a critical period during early in life determines the vascular aging phenotype in mice by altering miRNA-mediated post-transcriptional gene regulation.

IGF-1 is a critical regulator of development; yet, circulating levels of IGF-1 levels are highly variable during puberty (range: from ~100 to 800 ng/mL) (Bidlingmaier et al. 2014; Sorensen et al. 2012). In children with short stature, the prevalence of primary IGF-1 deficiency reaches 20 %. The significant variability in peripubertal IGF-1 levels is largely attributed to environmental factors, including socioeconomic status and diet. Protein intake is a key determinant of circulating IGF-1 levels in humans (Fontana et al. 2008), and clinical studies emphasize that nutritional deficiency associated with poverty (which affects over 8 million children in the USA) is a critical factor in the alarming incidence of peripubertal IGF-1 deficiency. Taken together, developmental IGF-1 deficiency and its long-term consequences are significant public health concerns, which affect millions of individuals in addition to those with rare genetic conditions of inherited IGF-1 deficiency.

In invertebrate model organisms, disruption of the insulin/IGF-1 pathway during development was reported to regulate lifespan and/or delay age-related pathophysiological alterations (Kimura et al. 1997). In mammals, the loss of insulin signaling during development is lethal. In recent years, the concept has emerged that alterations in developmental IGF-1 levels in mammals can also regulate aging processes, conferring both anti- and pro-aging effects later in life in an organ system-specific manner (Leiser and Miller 2010; Maynard and Miller 2006; Murakami et al. 2003; Nieves-Martinez et al. 2010; Page et al. 2009; Panici et al. 2010; Ramsey et al. 2002; Sadagurski et al. 2015; Salmon et al. 2005; Sonntag et al. 2005a; Ungvari et al. 2010, 2011; Wang

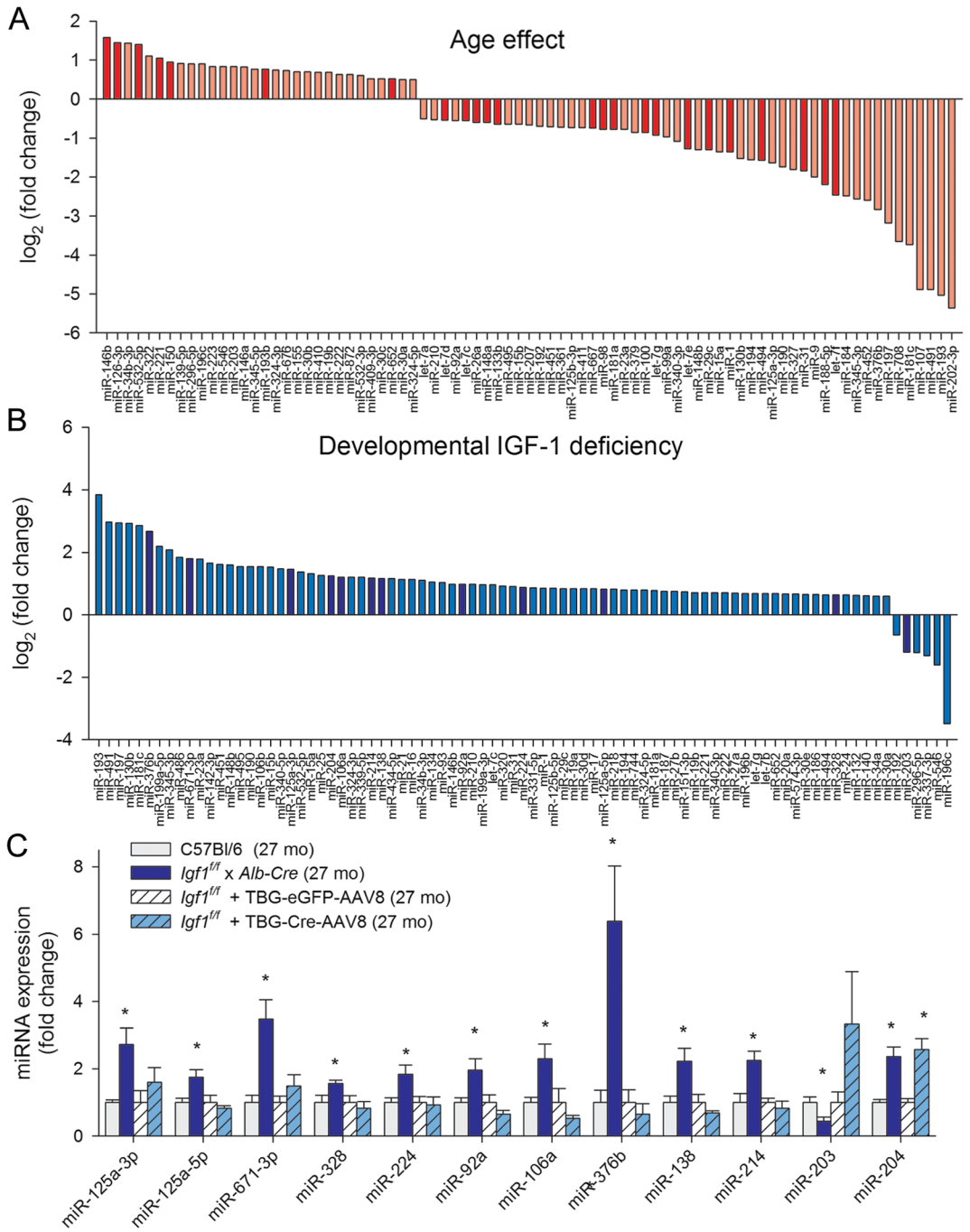


Fig. 2 Changes in miRNA expression profile in aortas associated with developmental IGF-1 deficiency and aging. **a**–**b** Effects of age (**a**) and developmental IGF-1 deficiency (**b**) on aortic miRNA expression. The *y axis* represents the average $\log_2$ fold change in miRNA expression levels in aortas derived from aged (27 months old) *Igf1*$^{f/f}$ *x* Alb-cre mice and aged (27 months old) control mice, relative to the corresponding control values. Significant ($p < 0.05$) changes are highlighted. The *x-axis* indicates the miRNA rank from the most upregulated to the most downregulated. $n = 5$–9 for each data point. **c** Comparison of the effects of developmental IGF-1 deficiency and adult-onset, post-pubertal IGF-1 deficiency. The expression of selected miRNAs significantly dysregulated in aortas of *Igf1*$^{f/f}$xAlb-cre mice was analyzed in aortas of *Igf1*$^{f/f}$ + AAV8-TBG-Cre mice by qPCR. Data are normalized to the mean miRNA expression in the aorta of the respective aged control group and are expressed as mean ± SEM (*$p < 0.05$). The data shows that developmental IGF-1 deficiency and adult-onset IGF-1 deficiency differentially alter miRNA expression in the mouse aorta

Table 2 GO terms enriched among miRNAs differentially expressed with age in the aorta

GO terms enriched among miRNAs differentially expressed with age in the aorta
Extracellular matrix
Chromatin silencing
Rab protein signal transduction
Signal transduction
Endopeptidase activity
Rac GTPase binding
Activation of protein kinase activity
Negative regulation of cyclin-dependent protein kinase activity
Chromatin DNA binding
Microtubule
Regulation of transcription
Cell migration
Endosome membrane
Intracellular protein transport
Negative regulation of cell death
Cytokine-mediated signaling pathway
Positive regulation of GTPase activity
Endosome
Stress-activated protein kinase signaling cascade
Regulation of mitotic cell cycle
Protein tyrosine/serine/threonine phosphatase activity
Positive regulation of protein targeting to mitochondrion
Cytokine production
Negative regulation of extrinsic apoptotic signaling pathway
Core promoter proximal region sequence-specific DNA binding
Regulation of protein kinase activity
Negative regulation of ERK1 and ERK2 cascade

At least one gene annotated with the GO category listed is targeted by miRNAs that are differentially regulated in the aged mouse aorta. Significance ($p < 0.05$) was determined by Fisher's exact test

Table 3 GO terms enriched among miRNAs differentially expressed with developmental IGF-1 deficiency in the aorta

GO terms enriched among miRNAs differentially expressed with developmental IGF-1 deficiency
Pattern recognition receptor signaling pathway
Production of miRNAs involved in gene silencing by miRNA
RISC-loading complex
Extracellular matrix
Blood vessel remodeling
Micro-ribonucleoprotein complex
miRNA loading onto RISC involved in gene silencing by miRNA
Pre-miRNA binding
Negative regulation of translation involved in gene silencing by miRNA
RISC complex
RNA polymerase II transcription factor binding
Negative regulation of cell proliferation
Pre-miRNA processing
miRNA binding
Transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding
SMAD binding
Regulation of transforming growth factor beta receptor signaling pathway
Endoplasmic reticulum membrane
Positive regulation of protein kinase activity
Regulation of transcription from RNA polymerase II promoter
Positive regulation of receptor-mediated endocytosis
Cytoplasmic mRNA processing body
RNA polymerase II transcription coactivator activity
Regulation of actin cytoskeleton organization
Regulation of protein localization
Phospholipid translocation
Negative regulation of microtubule depolymerization
Adaptive immune response
Positive regulation of apoptotic signaling pathway
Frizzled binding
mRNA polyadenylation
Protein tyrosine phosphatase activity
Cellular response to cAMP
Protein localization to cell surface
PDZ domain binding
Transcription factor complex
Vesicle organization
Negative regulation of BMP signaling pathway
Negative regulation of extrinsic apoptotic signaling pathway
Negative regulation of transforming growth factor beta receptor signaling pathway

and Miller 2012). Although early studies proposed that developmental IGF-1 deficiency contributes to the extension of lifespan in Ames dwarf mice and Snell dwarf mice (Panici et al. 2010), recent studies demonstrate that mice with isolated developmental endocrine IGF-1deficiency do not exhibit a longevity phenotype (Sonntag and Aspole, 2016, in preparation). Yet, in the same model, developmental endocrine IGF-1deficiency has been linked to marked alterations in healthspan (Ashpole et al. 2015). Interestingly, patients with Laron syndrome (congenital IGF-1 deficiency caused by

Table 3 (continued)

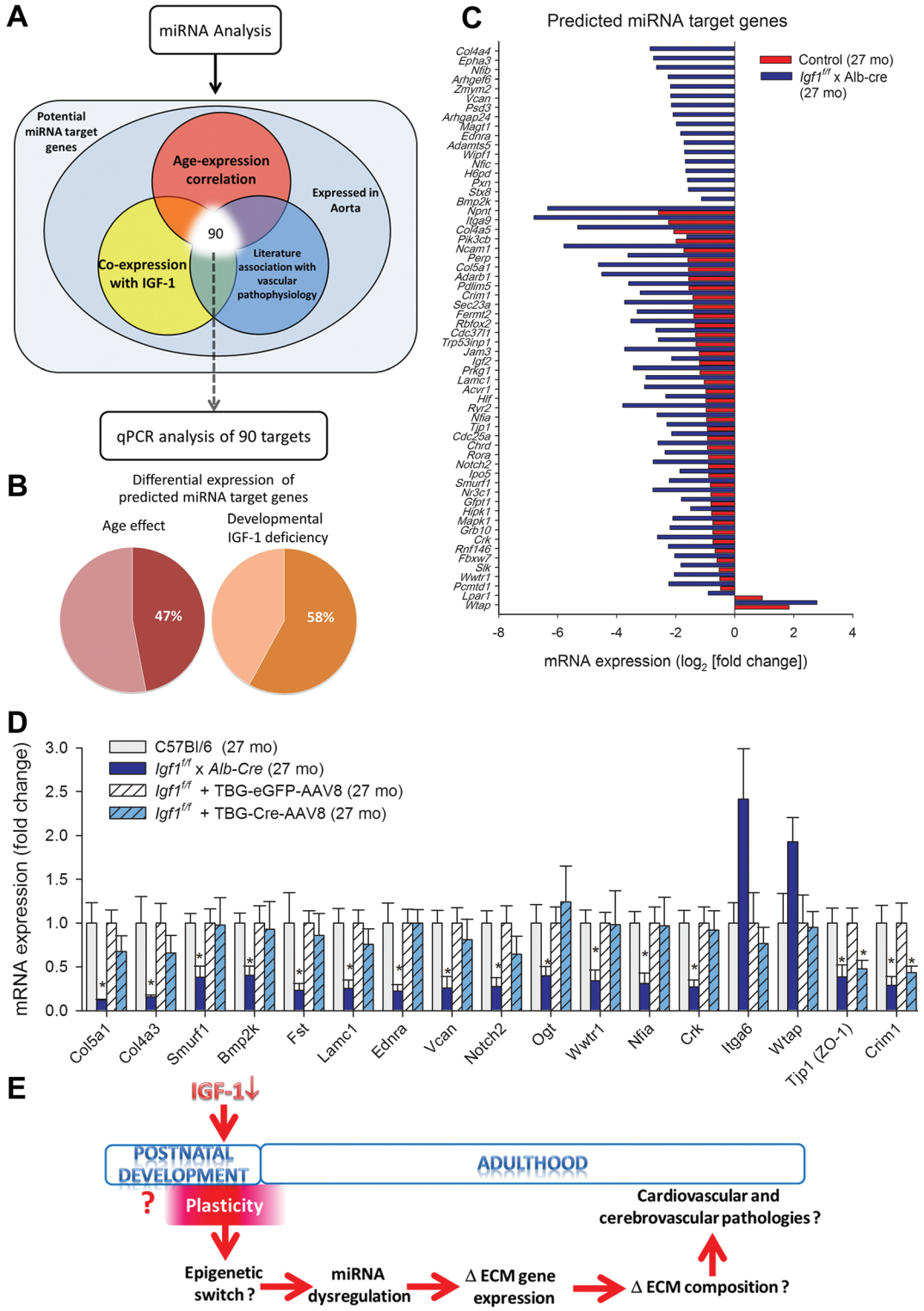
GO terms enriched among miRNAs differentially expressed with developmental IGF-1 deficiency
Poly(A) RNA binding
Glycoprotein binding
Chromatin DNA binding
Peptidyl-tyrosine dephosphorylation
Chaperone-mediated protein folding
Core promoter proximal region sequence-specific DNA binding
Sequence-specific DNA binding
Metallopeptidase activity
Transcription regulatory region DNA binding
Histone deacetylase binding
Endocytic vesicle
Negative regulation of translation
Positive regulation of gene expression
Single-stranded RNA binding
Endosome membrane
mRNA export from nucleus
Cytoskeleton
Intracellular protein transport
Integral component of membrane
Regulation of cell proliferation
Positive regulation of gene expression
Positive regulation of JNK cascade
Membrane raft
Cell differentiation
Vesicle
Transcription factor binding
Plasma membrane
Angiogenesis
Sequence-specific DNA binding
Negative regulation of transcription

At least one gene annotated with the GO category listed is targeted by miRNAs that are differentially regulated in the aorta of mice with developmental IGF-1 deficiency. Significance ($p < 0.05$) was determined by Fisher's exact test

primary GH insensitivity), who do not exhibit a longevity phenotype, seem to be protected against cancer at old age (Guevara-Aguirre et al. 2011; Steuerman et al. 2011), yet, are also affected by organ-specific symptoms of accelerated aging including osteoporosis, cognitive impairment, and marked obesity (Laron et al. 1999). Additionally, rodent models with developmental IGF-1 deficiency exhibit organ-specific signs of accelerated aging in the central nervous system and the musculoskeletal system (Ekenstedt et al. 2006; Sonntag et al. 2013).

Fig. 3 **a** Scheme illustrating the selection strategy adopted to predict putative biological targets of differentially regulated miRNAs for qPCR analysis (see Methods). **b** Percentage of predicted miRNA target genes whose aortic expression is significantly changed with developmental IGF-1 deficiency and age. **c** qPCR data showing the effect of developmental IGF-1 deficiency and aging on aortic mRNA expression of predicted biological targets of differentially regulated miRNAs. **d** Comparison of aortic expression of selected miRNA targets (qPCR data) shows that developmental IGF-1 deficiency (*Igf1*$^{f/f}$ *x* Alb-cre) and adult-onset IGF-1 deficiency (*Igf1*$^{f/f}$ + TBG-iCre-AAV8) differentially alter expression of genes related to extracellular matrix homeostasis and maintenance of vascular structural integrity. Data are mean ± SEM. *$p < 0.05$. **e** Proposed model for epigenetic mechanisms induced by IGF-1 in a critical peripubertal time window impacting vascular health later in life. The scheme depicts preadult periods of adaptive plasticity in the transition between juvenility to adolescence and to adulthood. This transition between developmental stages, which is governed in part by IGF-1, determines cardiovascular health span(Csiszar et al. 2008; Reddy et al. 2014; Sonntag et al. 2005a) and establishes longevity(Panici et al. 2010). We predict that persistent epigenetic mechanisms, including miRNA dysregulation and consequential alterations in extracellular matrix homeostasis contribute to the continued effects of the peripubertal IGF-1 surge later in life

Vascular endothelial and smooth muscle cells abundantly express IGF1R and are more sensitive to IGF-1 than to insulin (Chisalita and Arnqvist 2004; Chisalita et al. 2009; Johansson et al. 2008). Several lines of evidence suggest that normal developmental IGF-1 levels promote vascular health later in life (Ungvari and Csiszar 2012). Epidemiological studies demonstrate that poverty and malnutrition in adolescent children, which is known to be associated with low IGF-1 levels, increases risk for cerebrovascular diseases later in life (Forsdahl 1978; van Abeelen et al. 2012). Further, shorter stature, which is often a consequence of lower-than-normal developmental levels of IGF-1, has been associated with significantly increased risk of coronary heart disease and stroke (Eriksson et al. 2000; Goldbourt and Tanne 2002; Parker et al. 1998). Importantly, in Lewis dwarf rats, restoration of IGF-1 levels in a critical time window of ~10 weeks around puberty was also shown to increase lifespan by delaying a specific age-related vascular pathology—spontaneous intracerebral hemorrhages (Sonntag et al. 2005a, 2013). In the present study, we found that aged *Igf1*$^{f/f}$xAlb-cre mice exhibited significant endothelial dysfunction and vascular


A
miRNA Analysis
Potential miRNA target genes
Age-expression correlation
Expressed in Aorta
90
Co-expression with IGF-1
Literature association with vascular pathophysiology
qPCR analysis of 90 targets
B
Differential expression of predicted miRNA target genes
Age effect
Developmental IGF-1 deficiency
47%
58%
C
Predicted miRNA target genes
Control (27 mo)
Igf1f/f x Alb-cre (27 mo)
Col4a4
Epha3
Nfib
Arhgef6
Zmym2
Vcan
Psd3
Arhgap24
Magt1
Ednra
Adamts5
Wipf1
Nfic
H6pd
Pxn
Stx8
Bmp2k
Ngnt
Itga9
Col4a5
Pik3cb
Ncam1
Perp
Col5a1
Adarb1
Pdlim5
Crim1
Sec23a
Fermt2
Rbfox2
Cdc37l1
Trp53inp1
Jam3
Igf2
Prkg1
Lamc1
Acvr1
Hlf
Ryr2
Nfia
Tjp1
Cdc25a
Chrd
Rora
Notch2
Ipo5
Smurf1
Nr3c1
Gfpt1
Hipk1
Mapk1
Grb10
Crk
Rnf146
Fbxw7
Slk
Wwtr1
Pcmtd1
Lpar1
Wtap
-8
-6
-4
-2
0
2
4
mRNA expression (log2 [fold change])
D
C57Bl/6 (27 mo)
Igf1f/f x Alb-Cre (27 mo)
Igf1f/f + TBG-eGFP-AAV8 (27 mo)
Igf1f/f + TBG-Cre-AAV8 (27 mo)
mRNA expression (fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Col5a1
Col4a3
Smurf1
Bmp2k
Fst
Lamc1
Ednra
Vcan
Notch2
Ogt
Wwtr1
Nfia
Crk
Itga6
Wtap
Tjp1 (ZO-1)
Crim1
E
IGF-1↓
POSTNATAL DEVELOPMENT
ADULTHOOD
?
Plasticity
Cardiovascular and cerebrovascular pathologies ?
Epigenetic switch ?
miRNA dysregulation
Δ ECM gene expression
Δ ECM composition ?

oxidative stress and were not protected from the adverse vascular effects of aging (Fig. 1). Previous studies in human Laron syndrome patients (Guevara-Aguirre et al. 2011), Ames dwarf mice (Csiszar et al. 2008), mice harboring a liver-specific *Igf1* deletion (Troncoso et al. 2012) and Lewis dwarf rats (Bailey-Downs et al. 2012b; Cittadini et al. 1997; Longobardi et al. 2000; Ungvari et al. 2010) also show that developmental IGF-1 deficiency compromises cardiovascular health in adulthood. The available data suggest that developmental IGF-1 deficiency also exerts detrimental effects on stress resistance pathways, inflammatory processes and/or changes in structural characteristics of the vasculature later in life (Bailey-Downs et al. 2012b; Csiszar et al. 2008; Reddy et al. 2014; Ungvari et al. 2010). Collectively, our present findings and the aforementioned data from the literature do not support the often-cited hypothesis that developmental GH/IGF-1 deficiency exerts universal anti-aging effects (Panici et al. 2010).

To our knowledge, this is the first study to demonstrate that developmental IGF-1 deficiency elicits persisting late-life changes in miRNA expression profile in the vasculature (Fig. 2). These findings raise the possibility that changes in post-transcriptional control of expression of genes critical targets for vascular health underlie the late-life cardiovascular effects of developmental IGF-1 deficiency. The available evidence supports the concept that a link exists between circulating IGF-1 levels and miRNA expression (Bake et al. 2014; Bates et al. 2010; Fenn et al. 2013; Marino et al. 2010; Victoria et al. 2015). Demonstration of IGF-1-dependent changes in miRNA biology in the vasculature is particularly important (Bonauer et al. 2009; Chen et al. 2015; Doebele et al. n.d.; Hergenreider et al. 2012; Kim et al. 2014; Kuehbacher et al. 2007; Leung et al. 2013; Lovren et al. 2012; O'Rourke and Olson 2011; Rotllan et al. 2013; Stellos and Dimmeler 2014; Weber et al. 2014; Zampetaki et al. 2014) as changes in miRNA expression have been causally linked to the development of cardiovascular aging phenotypes (Boon et al. 2013; Csiszar et al. 2014; Ungvari et al. 2013) and the pathogenesis of cardiovascular diseases (Ono et al. 2011).

The mechanisms by which developmental IGF-1 deficiency alters miRNA expression that persists later in life are presently unknown. Recent studies showed changes in developmental IGF-1 levels during a critical time window in Lewis dwarf rats (Ungvari et al. 2011) and Snell dwarf mice ($Pit1^{dw/dw}$, which are phenotypically identical to Ames dwarf mice) (Panici et al. 2010) elicits long-lasting changes in cellular phenotypes, which persists in cell culture. These findings are consistent with the concept that changes in developmental IGF-1 levels result in epigenetic modifications to the genome. Recent studies have demonstrated that epigenetic mechanisms, including DNA methylation and histone modification, not only regulate the expression of protein-encoding genes, but also miRNAs, such as miR-203 (Sato et al. 2011). In that regard, it is significant that miR-203 is among the miRNAs selectively regulated by developmental IGF-1 deficiency. Further studies are warranted to test experimentally the role of IGF-1-mediated epigenetic regulation of miRNAs in the vasculature.

Dysregulation of miRNA pathways with developmental IGF-1 deficiency likely have important pathophysiological consequences in the cardiovascular system (Table 2). miRNA-dependent pathways have been shown to regulate multiple aspects of cellular physiology relevant for vascular aging, including angiogenesis (Kuehbacher et al. 2007; Suarez et al. 2007, 2008; Yang et al. 2005), structural integrity of the vessels, replicative senescence (Menghini et al. 2009; Vasa-Nicotera et al. 2011), mechanotransduction (Wu et al. 2011), NO production (Suarez et al. 2007; Wu et al. 2011), endothelial apoptosis (Asada et al. 2008), and inflammation (Suarez et al. 2007). Among the miRNAs whose expression is regulated by developmental IGF-1 deficiency, upregulation of miR-125a-5p has been linked to impaired angiogenesis and endothelial dysfunction (Che et al. 2014), endothelial apoptosis (Svensson et al. 2014), and dysregulation of endothelial tight junctions (Reijerkerk et al. 2013). miR-92a promotes atherosclerosis, endothelial dysfunction (Loyer et al. 2014), and neointima formation (Daniel et al. 2014). miR-126 is a biomarker of clinical atherosclerosis (Kim et al. 2015). miR-376b was reported to inhibit angiogenesis by targeting the VEGFA/Notch1 signaling pathway (Li et al. 2014). A functional link between upregulation of miR-138 and endothelial dysfunction has also been proposed (Sen et al. 2014).

Changes in miRNA expression induced by developmental IGF-1 deficiency likely also play important functional roles in impairing the structural integrity of the vessels, targeting components of the extracellular matrix. Accordingly, miR-328 is a negative regulator of collagen (*Col1a1*) expression (Rutnam et al. 2013; Rutnam and Yang 2012). miR-21 (Rutnam et al. 2013)

and miR-29 (Rutnam et al. 2013) also target collagens, whereas miR-671 downregulates fibronectin (Rutnam and Yang 2012). A link between miR-125a-5p (Rutnam et al. 2013) and impaired synthesis of extracellular matrix has been also documented. Changes in extracellular matrix synthesis and remodeling in the vascular wall during atherosclerosis, development of aneurysms, and the pathogenic processes leading to vascular ruptures (aorta dissection, hemorrhagic stroke, cerebral microhemorrhages) are governed by a wide range of growth factors and cytokines. These autocrine/paracrine mediators and their receptors can also be regulated by miRNAs. Accordingly, miR-224 was reported to modulate extracellular matrix synthesis via regulation of connective tissue growth factor (Chen et al. 2014).

To better understand the pathophysiological relevance of late-life miRNA dysregulation induced by developmental IGF-1 deficiency, we analyzed expression of predicted targets of altered miRNAs known to be involved in maintenance of structural and functional integrity of the vascular system. Using a novel computational approach, we identified miRNA target genes that associate with IGF-1 deficiency, aging, and vascular pathophysiology. Our method accurately predicted genes whose expression was dysregulated in mice with developmental IGF-1 deficiency (Fig. 3). We found that many age-related changes in vascular expression of miRNA target genes were exacerbated in mice with developmental IGF-1 deficiency (Fig. 3b). Further, developmental IGF-1 deficiency and adult-onset IGF-1 deficiency differentially altered expression of the predicted miRNA target genes in the mouse aorta (Fig. 3c). The aforementioned findings provide strong support for the concept that early-life changes in the hormonal milieu have significant impact on cardiovascular health-span later in life, accelerating vascular aging.

Importantly, we confirmed that the expression of multiple extracellular matrix-related genes, including collagen-encoding genes, were preferentially downregulated in mice with developmental IGF-1 deficiency (Fig. 3c). These results extend previous findings demonstrating that developmental IGF-1 deficiency promotes structural impairment and extracellular matrix remodeling in vessels of aged Lewis dwarf rats, increasing their propensity to spontaneous rupture (Sonntag et al. 2005a). Interestingly, developmental IGF-1 deficiency is also associated with decreased collagen expression in the cardiovascular system of Ames dwarf mice (Helms et al. 2010). Future studies are evidently needed to experimentally dissect the IGF-regulated pathways regulating extracellular matrix homeostasis and vascular remodeling (Bruel and Oxlund 2002; Shai et al. 2010; Ungvari and Csiszar 2012) in the models used.

In addition to collagen encoding genes, we found that other factors controlling vascular integrity are also downregulated in mice with developmental IGF-1 deficiency (Fig. 3d). Bone morphogenetic proteins are important regulators of extracellular matrix homeostasis. Interestingly, our data suggest that developmental IGF-1 deficiency results in dysregulation of BMP signaling pathways in the vascular wall. We found that developmental IGF-1 deficiency results in downregulation of the adapter protein Crk, which is involved in growth regulation, cell migration, and cell adhesion. It is significant that genetic deletion of Crk results in increased vascular fragility (Park et al. 2006). Vascular expression of paxillin was also downregulated in mice with developmental IGF-1 deficiency. Paxillin is expressed at focal adhesions, which adhere the cytoskeleton of smooth muscle cells to the extracellular matrix in the vascular wall and thereby contribute to the tensile strength of the vasculature. We found that developmental IGF-1 deficiency alters the expression of laminin, a major constituent of basement membranes dysregulated in aging (Gavazzi et al. 1995) and α6 integrin, a specific laminin receptor. Both aging and developmental IGF-1 deficiency tend to upregulate Wilms' tumor 1-associating protein (WTAP), a nuclear protein that interacts with the Wilms' tumor 1 tumor suppressor gene product (WT1). WTAP is a newly discovered component of the m6 A methyltransferase complex, which plays a critical role in epitranscriptomic regulation of RNA metabolism (Ping et al. 2014). Recent studies show that WTAP inhibits the proliferation of vascular smooth muscle cells and endothelial cells and promotes apoptosis, regulating vascular remodeling (Small et al. 2006, 2007). During development of the vasculature IGF-1 was shown to downregulate WTAP, which is necessary for IGF-1 to confer its antiapoptotic effects, regulating smooth muscle cell fate (Small and Pickering 2009). Another factors affected by developmental IGF-1 deficiency are endothelin receptor A, versican and O-GlcNAc transferase (Ogt), and Wwtr1. Endothelin receptor A is important for vascular development and maintenance of vascular integrity (Donato et al. 2014). Importantly, genome-wide association studies identify EDNRA as a possible factor in the

pathogenesis of intracranial aneurysms (Low et al. 2012; Yasuno et al. 2011). The proteoglycan versican has a key role in extracellular matrix assembly and contributes to the pathogenesis of intracranial aneurysms (Sathyan et al. 2014). Changes in the O-linked-N-acetylglucosamine (O-GlcNAc) modification of cytoplasmic and nuclear proteins, catalyzed by O-GlcNAc transferase, regulates a wide range of cellular functions and have been associated with a number of age-related diseases (Fulop et al. 2008). Wwtr1 (TAZ) is a transcriptional coactivator that links mechanosensing of extracellular matrix stiffness to activity of nuclear transcription factors in vascular cells (Dupont et al. 2011). Interestingly, while the aforementioned miRNA target genes were uniquely regulated by developmental IGF-1 deficiency, other targets, such as TJP1 (ZO-1, which plays a role in assembly of tight junctions, regulating endothelial permeability and vascular development) and CRIM1 (which regulates vascular stability and angiogenesis) appear to be affected by post-pubertal IGF-1 status.

Taken together, out of the results of the miRNA profiling experiments and the target validations studies the concept emerges that IGF-1 deficiency during a critical period through development impacts extracellular matrix biology and smooth muscle phenotype later in life via miRNA-regulated pathways, thereby altering the composition and organization of the tissue microenenvironment and contributing to the pathogenesis of age-related vascular diseases. In support of this concept, there is growing evidence that in humans and experimental animals, the origins of pathologies associated with structural weakening of the vascular wall (e.g., intracerebral hemorrhages) occur during puberty, a time of rapid changes in the cerebral circulation and structural brain development (Blakemore et al. 2010; Blanton et al. 2012; Giedd et al. 2006; Goddings et al. 2014; Manz et al. 1979; Peper et al. 2011; Satterthwaite et al. 2014).

Limitations of the study

There are important limitations of our study, including the limited endpoints tested. Further studies are warranted to assess vascular miRNA expression profile and their predicted targets in young $Igf1^{f/f}$ x Alb-cre mice and in aged $Igf1^{f/f}$ x Alb-cre mice with peripubertal IGF-1 replacement. Our recent studies suggest that the consequences of a loss of circulating IGF-1 on vertebral bone aging are different in male and female mice due to compensatory changes in IGF-1 signaling (Ashpole et al. 2015). Thus, future studies should determine whether late-life effects of developmental IGF-1 deficiency on vascular health are also sex-specific. There are studies suggesting that IGF-1 deficiency determines intima-media thickness in human patients (Colao et al. 2004); thus, future studies should also determine how experimental IGF-1 deficiency affects neointima formation in our models.

Conclusions

The findings of the present study provide additional experimental evidence in support of the concept that IGF-1 levels in a critical period early in life influence vascular health later in life (Fig. 3e). Among the possible diverse developmental epigenetic processes regulated by IGF-1, our data provide evidence for persistent changes in miRNA-mediated post-transcriptional gene regulation in the vasculature. Importantly, our findings suggest that developmental IGF-1 levels significantly impact post-transcriptional regulation of expression of genes regulating structural integrity of the vasculature, including components of the extracellular matrix. Future studies should fully elucidate the mechanistic effects of developmental IGF-1 levels on the pathogenesis of specific vascular diseases that involve remodeling/degradation of the extracellular matrix (including intracerebral hemorrhages, atherosclerosis, aneurysm), to characterize the peripubertal time window for the late-life effects of developmental IGF-1 on vascular healthspan and to study the contribution of individual miRNAs or miRNA clusters regulated by developmental IGF-1 deficiency in controlling gene expression that underlie extracellular matrix remodeling and microvascular aging.

Acknowledgments This work was supported by grants from the American Heart Association (ST, AC, MNVA, ZT and ZU), the Oklahoma Center for the Advancement of Science and Technology (to AC, ZU, WES), the Oklahoma IDeA Network for Biomedical Research Excellence (to AC), the NIH (AG031085 to AC; AT006526 to ZU; AG038747 and NS056218 to WES and AC; 5U54GM104938 to JW), the Ellison Medical Foundation (to WES), and the Arkansas Claude Pepper Older Americans Independence Center at University of Arkansas Medical Center (to JW and ZU; P30 AG028718).

Compliance with ethical standards All procedures were approved by and followed the guidelines of the Institutional Animal

Care and Use Committee of OUHSC in accordance with the ARRIVE guidelines.

Conflict of interest The authors declare that they have no conflict of interest.

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