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MicroRNAs in the growth plate are responsive to nutritional cues: association between miR-140 and SIRT1☆,☆☆

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Abstract

MicroRNAs (miRNAs) have been reported to be involved in a variety of functions, including skeletal development and longitudinal growth. The aim of this study was to investigate the role of miRNAs in food-restriction-induced growth attenuation and nutrition-induced catch-up growth in the epiphyseal growth plate (EGP). Prepubertal rats were fed *ad libitum* or were subjected to 40% food restriction for 10 days followed by a renewal of the regular food supply. At sacrifice, tibial EGPs were excised, and the total RNA was extracted and loaded on miRNA microarrays. The miRNA microarray yielded more than 400 miRNAs that are expressed in the EGP of mature animals. Results were confirmed by quantitative polymerase chain reaction. Chondrocyte-specific miR-140-3p showed the highest expression in the mature EGP, and it was one of the few miRNAs that were significantly reduced following nutrition restriction. Changes in predicted miRNA targets were then followed with Western immunoblotting. Direct binding was demonstrated using exogenous miRNA, the 3′UTR of the target mRNA and a luciferase reporter assay. Nutrition restriction induced an increase in the level of the miR-140-3p target, NAD+-dependent SIRT1. This study is the first to show that SIRT1 and miRNAs expressed in the mature EGP are responsive to nutritional cues. Nutrition-induced epigenetic regulation of growth activates two parts of the epigenetic world — miRNAs and histone deacetylases — that are interconnected. Deciphering the role of epigenetic regulation in growth may open a new era of research and pave the way for the development of new treatments for children with growth disorders.

Keywords: microRNA; Growth plate; Catch-up growth; miR-140; SIRT1

1. Introduction

By the end of the Human Genome Project, researchers recognized that a large part of the genome is not translated into proteins. It has since became apparent that this noncoding, so-called "junk" DNA is highly relevant to the control of gene expression and maintenance of genomic stability and serves as the site of action of epigenetic regulation. Epigenetics is defined as changes in gene function caused by mechanisms other than changes in the genomic DNA sequence, for example, chromatin structure remodeling together with chemical modifications of DNA and associated proteins, such as histones, as well as gene-regulating activities by small (18–24 nucleotides long) non-protein-coding RNAs termed microRNAs (miRNAs) [1]. miRNAs negatively regulate gene expression at the posttranscriptional level. Each miRNA can regulate several mRNA transcripts, and each mRNA may be regulated by one to several miRNAs [2,3]. miRNAs have been shown to play a role in many cellular processes, including adipocyte differentiation, insulin secretion, B-cell development, neural stem cell fate, immune function and skeletal development [4–10]. They are deregulated in numerous diseases including cancer, neurodegenerative diseases and autoimmunity [11–13].

Postnatal skeletal growth depends on an elaborate cascade of events that take place in the cartilaginous growth center of the long bones, called the epiphyseal growth plate (EGP), and culminate in endochondral ossification. The process of endochondral ossification involves the proliferation of early chondrocytes and production of columns of flat proliferating cells followed by ingress of water and increase in volume and metabolic activity until maturation of the cells into hypertrophic chondrocytes. At this stage, the cells cease dividing, secrete extracellular matrix and undergo programmed cell death. This is accompanied by vascular invasion, mineralization of the extracellular matrix and replacement of the cartilage scaffold with bone tissue.

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Longitudinal growth at the EGP is controlled by both local factors and systemic factors, namely, local growth factors, components of the extracellular matrix and hormones. It can be impaired by chronic illness, malnutrition and other pathological conditions. In most cases, when the growth-inhibiting condition is resolved, spontaneous catch-up (CU) growth occurs. CU growth is defined as "height velocity above the normal statistical limits for age and/or maturity during a defined period of time, following a transient period of growth inhibition" [14]. CU growth is probably an endogenic capability of the EGP [15].

Despite the enormous effort of pediatric endocrinologists, dieticians and research scientists to expand our understanding of the interaction of nutrition and linear growth in children, the exact mechanism by which the body signals the EGP to grow or attenuate growth is still unclear. In the last couple of years, our group has conducted a series of experiments to determine the factors underlying the association between nutrition and growth. Together with others (reviewed in Ref. [16]), we have identified the role of leptin in the regulation of linear growth [17–19], and we have shown that HIF1α, a transcription factor essential for EGP growth and development [20], is responsive to nutritional status [21].

Prompted by reports showing miRNA expression in cartilage [22], in the present study, we applied our system of robust growth changes to investigate the potential role of miRNAs in regulating growth attenuation and CU growth in the mature EGP. We found that miRNAs expressed in the mature EGP are affected by food restriction. Furthermore, we discovered an apparently direct association between two parts of the epigenetic regulatory mechanisms: miRNA and histone deacetylase (HDAC) of the sirtuin family, SIRT1. These findings may ultimately have important implications for the treatment of growth disorders in children.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, 24 days old, were purchased from Harlan (Jerusalem, Israel) and housed individually at the animal care facility of Felsenstein Medical Research Center. The rats were divided into two groups: one was given an unlimited amount of food (complete diet for rats and mice, 3.4 kcal/g, provided by Teklad, South Easton, MA, USA) (*ad libitum* group; AL), and the other was given 60% of the same chow (food-restricted group; RES); all animals had unlimited access to water. The 40% restriction was calculated on the basis of a previous study wherein animals were housed individually, and the amount of food consumed each day was measured, together with the animals' weight and weight gain, as described [21]. The food restriction was maintained for 10 days. At that point, the RES group was further divided into two groups: one was kept restricted, and the other was given normal chow *ad libitum* (CU group). After 1 day of refeeding, animals from all three groups (n=6 each) were sacrificed by CO_2 inhalation. Throughout the study, animals were observed daily, and all remained bright, alert and active, with no evidence of any disorder. The Tel Aviv University Animal Care Committee approved all procedures.

At sacrifice, both tibial EGPs from each animal were removed, isolated and stored at −70°C, creating a "pool" of growth plates for each group. It was necessary to work with pools of EGPs because of the tiny size of the EGP. The whole experiment was repeated five times. The results of each experiment were analyzed independently using quantitative polymerase chain reaction (qPCR) and Western immunoblots.

2.2. Antibodies

Anti-SIRT1 was obtained from Millipore (Billerica, MA, USA); anti-IGF-1 binding protein 7 (IGFBP7) and anti-IGF 1 receptor (IGF-1R) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA;) anti-HDAC4 was from Cell signaling (Danvers, MA, USA); anti-β-actin was obtained from Abcam (Cambridge, MA, USA); secondary fluorescent antibodies were obtained from LI-COR Biosciences (Lincoln, NE, USA).

2.3. Radioimmunoassay for serum IGF-1

IGF-1 levels were determined with a standard double-antibody radioimmunoassay kit with extraction (5600 ACTIVE IGF-1 IRMA; Diagnostic System Laboratories, Inc., DSL Inc., Webster, TX, USA) according to the manufacturer's instructions.

2.4. RNA extraction

Total RNA was extracted from the EGPs according to the acid guanidinium thiocyanate method [21]. All RNA samples were stored at −70°C. RNA concentration was assessed using Nanodrop (Nanodrop Technologies, Wilmington, DE, USA). The integrity of the RNA was confirmed by running samples on denaturing agarose gel and visualization under UV light after ethidium bromide staining.

2.5. Rosetta Genomics miRNA microarray

Total RNA was extracted from the EGP samples and hybridized to a Rosetta Genomics (Rehovot, Israel) proprietary microarray. In this microarray, probes are represented in triplicate, and 54 negative and positive controls are spotted in duplicate. Normalization was performed as previously described [23]. Briefly, triplicate spots were combined to produce one signal for each probe by taking the logarithmic mean of reliable spots. All data was log-transformed (natural base), and the analysis was performed in log-space. A reference data vector for normalization was calculated by taking the median expression level for each probe across all samples [23]. For each sample, a second-degree polynomial function F was found so as to provide the best fit between the sample data and the reference data. Remote data points ("outliers") were not used for fitting the polynomial function. For each probe in the sample, the normalized value (in log-space) is calculated from the initial value by transforming it with the polynomial function F found for this sample [23].

Quality assessment was based on similarity of a sample to the median of all samples and by a high dynamic range (>70 miRNA expressed a signal above 500). The procedure and analysis were performed at the company's facilities by company personnel and was repeated four times.

2.6. Reverse transcription (RT) and qPCR for mRNA

First-strand cDNA synthesis was performed with SuperScript III RTS First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) using 1 μg of total RNA according to the manufacturer's instructions. This step was preceded by Dnase I treatment (Promega, Madison, WI, USA). qPCR was performed with the ABI Prism 7000 Sequence Detection System [Applied Biosystems (ABI), Foster City, CA, USA]. According to the manufacturer's instructions, PCR reactions were done with specific FAM-labeled probes [TaqMan assay on demand for SIRT1 RN01428093_m1; IGFBP7: Mm00514987_m1; IGF-1-R RN01477918_m1 and Aconitase (Aco2): RN00667086_m1]. Aco2 was selected to serve as the internal control because it was previously found to have the least variability and greatest reproducibility in our system compared to other genes, such as 18S [21]. The following thermal cycling conditions were used: one cycle at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The probes and reaction mixture were obtained from ABI. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method [24]. Each sample was examined in triplicate.

2.7. RT and qPCR for miRNA

qPCR was performed with the 7000 Sequence Detection System (ABI). In the first step, 10 ng of the total RNA were mixed with 0.015nmol dNTPs, 50 U Multiscribe Reverse Transcriptase, 1.5 μl 10× RT buffer and 3.8 U RNAse inhibitor. Appropriate primers were added to yield the RT product. The procedure was performed with 1.33 μl of the RT product, Taqman Universal PCR Master mix and a specific FAM-labeled probe/primer set [TaqMan assay on demand for hsa-let-7e, no: 4373165; hsa-miR-196a, no: 4373104; hsa-miR-126, no:4395339, hsa-miR-140-3p, no: 4373138; U6B served as the internal control (RnU6B, no: 437338)]. These probes had the hsa-miR nomenclature, in accordance with the probes used in the Rosetta Genomics microarrays. The following thermal cycling conditions were used: one cycle at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The probes and reaction mixture were obtained from ABI. Analysis of the results was performed as described for mRNA.

2.8. Microdissection of the growth plate

Proximal tibial EGPs from the rats were embedded in OCT and stored at −70°C. Thereafter, 50-μm-thick longitudinal frozen sections were mounted on Superfrost/Plus microscope slides (Fisher Scientific, Waltham, MA, USA). The slides were thawed for 2 min; fixed serially in 70% ethanol, 100% methanol and 95% ethanol; stained in eosin; washed in 100% ethanol and dehydrated in xylene. Each step was conducted for 60 s at room temperature [21].

The proliferative and hypertrophic zones were separated from each section using an inverted microscope and a scalpel. A narrow buffer zone of chondrocyte cells between the proliferative and hypertrophic zones was discarded to avoid cross-contamination of the two zones. Using the microdissection method, we identified the cells according to both their morphology and location, which guarantees very high accuracy. For RNA isolation, at least 15 sections from each zone were pooled for each animal.

2.9. Protein extraction and determination

EGPs derived from tibial bones of rats from all groups were homogenized in a commercial extraction buffer supplemented with Benzonase (Sigma-Aldrich) and a protease inhibitor cocktail (Roche, Basel, Switzerland) in a 1:12 ratio. Protein concentration was determined, and samples containing an equal amount of proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western immunoblotting. Positive immunoreactivity was detected with the secondary fluorescent antibody (LI-COR) decorated with IRDye. Anti-β-actin was used as the reference.

2.10. Prediction of miRNA targets

There are many miRNAs, each with a large number of predicted targets. However, only a handful of targets have been experimentally confirmed. In the absence of a high-throughput experimental technique to determine the targets, several computational algorithms were developed to identify putative targets of miRNAs and, hence, their biological effects [to name a few: PicTar (http://pictar.mdc-berlin.de/), mirBase (http://microrna.sanger.ac.uk/targets/v4/) and TargetScan (http://www.targetscan.org)]. However, although each of these algorithms yields a large number of putative targets, the overlap is surprisingly low [25]. To narrow our search to relevant targets, we selected from the predicted-target algorithms only those targets that may be involved in growth or epigenetic regulation according to published data [22,26,27].

2.11. Construction of vectors and dual-luciferase activity assay

The binding recognition site for miR-140-3p localized in the 3′UTR of the SIRT1 gene was amplified from rat EGP, cut by the restriction enzymes *Xho*I and *Not*I (both from New England Biolabs, Beverly, MA, USA) and cloned into the 6273-bp vector psiCHECK-2 (Promega) immediately downstream of the Renilla luciferase gene (psiCHECK-SIRT1). In a similar manner, psiCHECK-IGFBP7 plasmid was constructed. The following primer sets were used to amplify the required region for PCR: for SIRT1 (located on Chr 20:25,668,033-25,688,196, according to http://www.ensembl.org/): forward (5′-3′) CCG CTC GAG CTC AAT TTC TGT TCT GCT G, reverse (3′-5′) ATA GTT TAG CGG CCG CTT AAG AGA CCT TTG CTT CTC CC; for IGFBP7 (Chr 14: 33,010,198-33,070,052): forward (5′-3′) CCG CTC GAG ACG ACA GTC ACC GAA ACA, reverse (3′-5′) ATA GTT TAG CGG CCG CTG CTG CGA GTC TGC TTA T. Site-directed mutagenesis was used to specifically mutate nucleotides 2–4 of miR-140-3p recognition site in the 3′UTR region of SIRT1 (Quickchange II site-directed mutagenesis kit; Agilent Technologies, La Jolla, CA, USA) according to the manufacturer's instructions) (psiCHECK-SIRT1-sdm).

Vectors containing miR-140 and miR-126 were prepared as described (miR-Vec-140; miR-Vec-126) [28]. In brief, pre-miR 140 and pre-miR-126 were amplified using the appropriate primers from genomic DNA. These fragments were inserted into the miR-Vec vector between the *Eco*RI and *Bam*HI sites. All vector sequences were validated by direct sequencing.

The human cell line HEK293 was grown to 70% confluence in 24-well plates and then co-transfected with three constructs: 10 ng of the pEGFP-C1 vector expressing a green fluorescent protein (GFP) and a total of 500 ng of the reporter vector with or without the relevant miR-Vec. Successful transfection was validated by following GFP expression under a fluorescent microscope. Only wells with >70% transfection efficiency were used for luciferase assays. Cells were harvested 72 h after transfection, and the luciferase activity assay was performed using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA). The relative luciferase activity was calculated as the ratio of the Renilla luciferase activity to the firefly luciferase activity and compared to cells transfected with the relevant psiCHECK vector alone. Data are shown as the mean±S.E. percentage of controls.

2.12. Statistical analysis

Results are expressed as mean±S.E. Differences between groups were analyzed by Student's *t* test or one-way analysis of variance. The statistically significant threshold was set at $P<.05$.

3. Results

3.1. Expression of miRNAs in the mature EGP

To investigate the role of miRNA in growth regulation, we profiled miRNA expression in the EGP of mature, 34-day-old rats. Numerous miRNAs were found to be expressed above the limit of a signal value of 500. The 50 with the highest expression and their possible role in development and growth regulation are presented in Supplemental Table 1 (Supplementary data, see supplementary Table 1 on the journal's website at www.jnutbio.com). qPCR was performed with probes for several highly expressed miRNAs (miR-140-3p, miR-126, miR-196a1 and let 7e), and their relative expression was verified (data not shown). The most highly expressed miRNA was found to be miR-140-3p, in agreement with a previous publication [22].

3.2. miRNA expression following food restriction

Microarray and PCR studies were repeated for total RNA extracted from the whole EGP of 34-day-old rats after 11-day diet of unlimited chow (AL group) or 60% chow (RES group) or 10 days of 60% chow followed by 1 day of normal chow *ad libitum* (CU group). Following food restriction, the expression of only a few miRNAs was significantly and consistently reduced (according to Rosetta Genomics arrays; later verified by qPCR in at least five independent *in vivo* experiments). Food restriction induced a reduced expression of four miRNAs: miR-140-3p, miR-126, miR-22 and miR-21. After 1 day of refeeding, the expression of miR-126 and miR-21 remained unchanged, whereas miR-22 and miR-140-3p expression tended to increase (Fig. 1).

We also examined the levels of two miRNAs that were highly expressed in the EGPs but seemingly unaffected by nutritional manipulation according to the microarray study: let-7e and miR-196a-1. The qPCR results revealed no significant effect of nutritional manipulation on the levels of these miRNAs, indicating that the reduction was not a general phenomenon (data not shown).

3.3. Microdissection analysis

The specific localization of the affected miRNAs in the EGP was studied by microdissection. All four miRNAs were found to be expressed in both the proliferative and hypertrophic zones. Interestingly, the food restriction induced a significant reduction in the level of these miRNAs only in the proliferative zone (data not shown).

3.4. Effect of nutritional manipulation on target levels in the EGP

To study the effect of nutrition on miRNA targets, we focused on miR-140-3p, which had the highest expression level in the mature EGPs. Among the predicted targets of miR-140-3p, we checked changes in the protein level of two HDACs: HDAC4, previously shown to be involved in chondrogenesis [22], and SIRT1, previously shown to be involved in the response to long-term calorie restriction in other systems [27]. As shown in Fig. 2, the protein level of HDAC4 was significantly reduced by calorie restriction, whereas its mRNA levels on Affymetrix chip increased slightly. By contrast, mRNA levels of

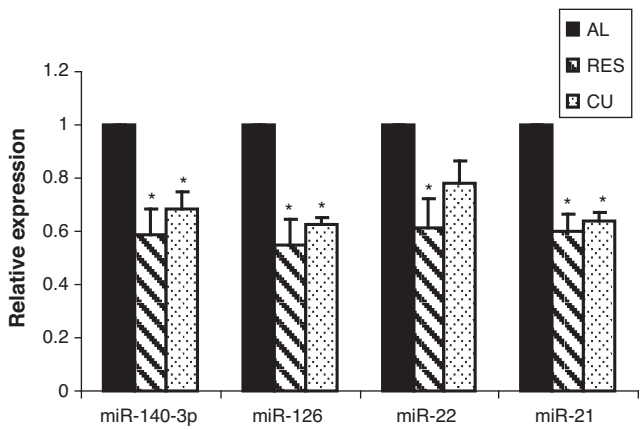

Fig. 1. Effect of food restriction on expression levels of miR- 140, miR-126, miR-22 and miR-21. qPCR was performed on RNA extracted from the EGP. Expression levels are presented relative to the AL group. Results given are the average of five separate experiments. *$P<.05$ compared with AL.

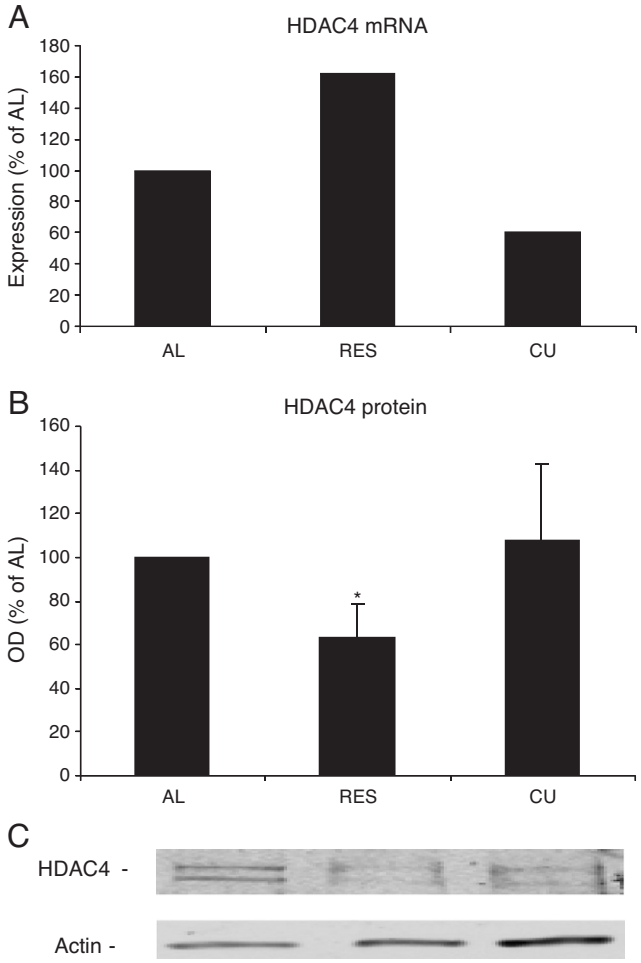

Fig. 2. Effect of food restriction on expression and protein levels of HDAC4. (A) Expression levels of HDAC4 mRNA from the expression microarray [21]. (B) Densitometric analysis of the Western immunoblots for HDAC4 protein (presented in C). Results are presented as percentage of the AL group. All data are given as mean±S.E. *P<.05 compared with AL. (C) Western immunoblots for HDAC4 with β-actin as reference. Results given are the average of three separate experiments.

SIRT1 showed no significant response to nutritional manipulation, but quantitative analysis of the immunoblots disclosed higher expression of SIRT1 in the RES group than the AL group (P<.05). In the CU group, the increase associated with food restriction was followed by a reduction already 1 day after refeeding (Fig. 3).

We further explored SIRT1 expression in the different zones of the EGP using microdissection. Higher expression of SIRT1 mRNA was found in the proliferative than the hypertrophic zone in all groups (Fig. 4A), in agreement with the immunohistochemistry results for the protein (Fig. 4B). SIRT1 was identified in both the nucleus and the cytoplasm of the cells.

3.5. Direct effect of miRNA-140-3p on the 3′UTR of SIRT1

To confirm the association between miR-140-3p and its putative target SIRT1, it was necessary to establish that the relevant binding site in the 3′UTR sequence is expressed by chondrocytes of the EGP and is not subject to alternative splicing. Indeed, we found that the 3′UTR of SIRT1, containing the putative binding site for miR-140-3p, is expressed by EGP chondrocytes (Fig. 5A). Next, a luciferase assay was established. Co-expression of miR-Vec-140 with psiCHECK-SIRT1 significantly reduced the luciferase activity. No significant effect on luciferase activity was noted when miR-Vec-140 was co-expressed with a mutated 3′UTR-containing vector, psiCHECK-SIRT1-sdm (Fig. 5B).

3.6. Effect of nutrition on IGFBP7

Next, we repeated the analysis for miR-126. Among the predicted targets of miR-126, we chose IGFBP7, known to be involved in cellular growth [26]. The mRNA levels of IGFBP7 were significantly reduced by calorie restriction, but the level of the protein was significantly

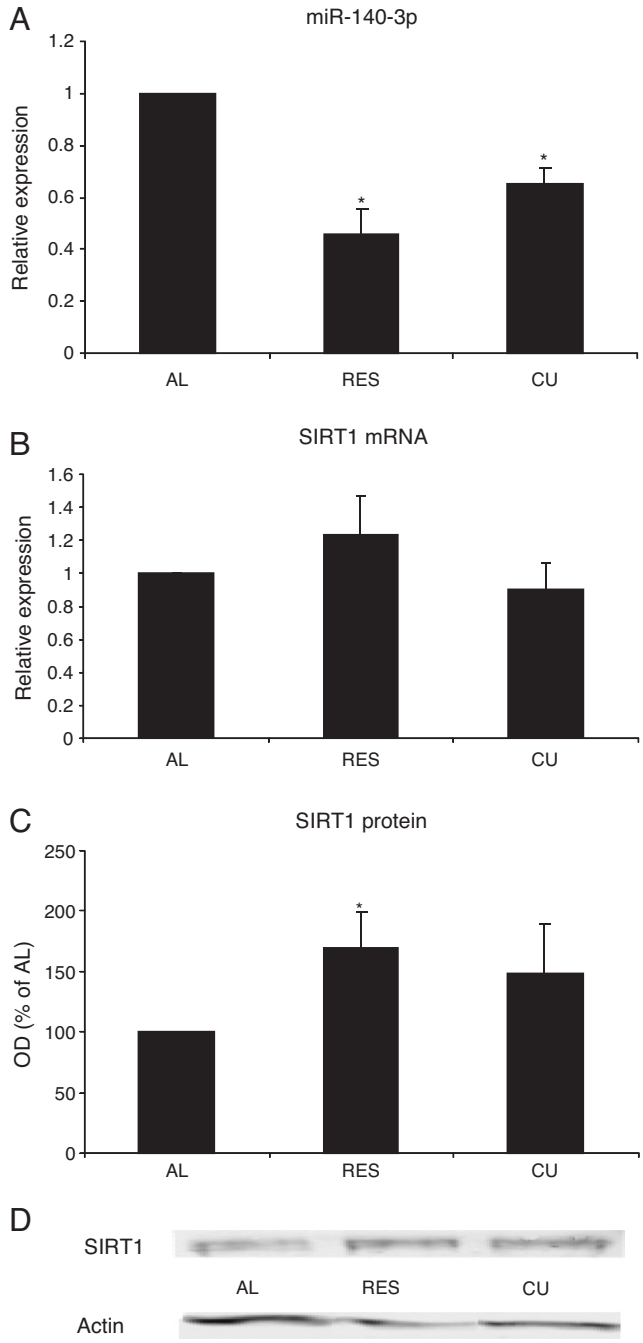

Fig. 3. Effect of food restriction on expression levels of miR-140-3p and its predicted target, SIRT1. (A) qPCR of miR-140-3p in the EGP. Expression levels are presented relative to the AL group. (B) qPCR of SIRT1 mRNA. (C) Densitometric analysis of Western immunoblots for SIRT1 protein (presented in D). Results are presented as percentage of AL. All data are given as mean±S.E. *P<.05 compared with AL. (D) Western immunoblots for SIRT1 with β-actin as reference. Results given are the average of three separate experiments.

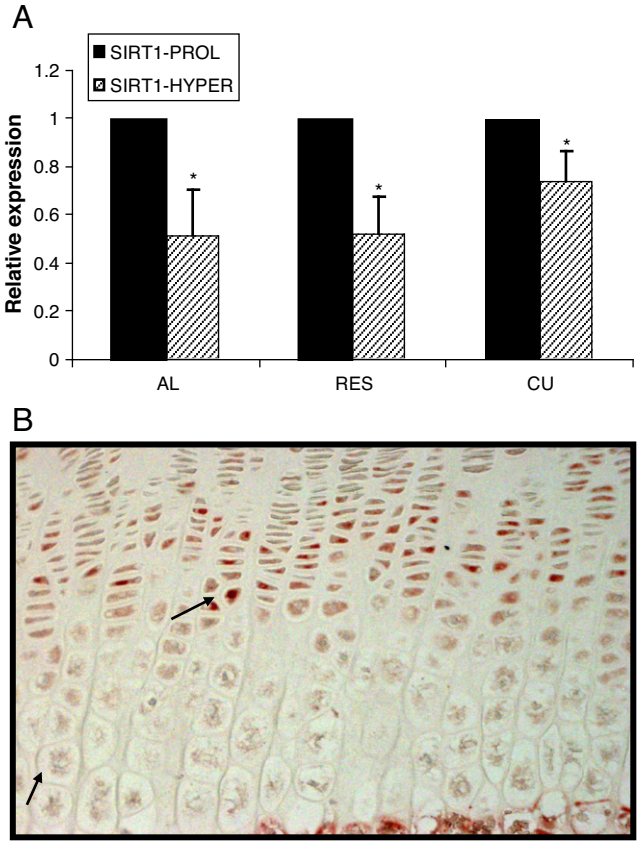

Fig. 4. Effect of food restriction on expression levels of SIRT1 in microdissected EGP. (A) qPCR of SIRT1 mRNA in the proliferative and hypertrophic zones of the proximal tibial EGP. *P<.05 compared with proliferative zone. (B) Immunohistochemical detection of SIRT1 in the proximal humeral EGP. Staining was located mainly in the transition and proliferative zones, and mild staining was detected in the hypertrophic zone (arrows indicate positive staining). Amplification ×20.

increased (P<.05). However, in contrast to the results for miR-140-3p and SIRT1, after 1 day of refeeding (CU group), the IGFBP7 protein expression returned to baseline, and the IGFBP7 mRNA level remained low. Although the 3′UTR region, containing the putative binding site for miR-126 on IGFBP7 mRNA, was found to be expressed by EGP chondrocytes on luciferase assay, the co-expression of miR-Vec-126 and psiCHECK-IGFBP7 failed to reduce luciferase activity (Supplementary figure 1). Thus, the discrepancy between the mRNA and protein levels in this case warrants further investigation at other regulatory levels.

3.7. Effect of nutritional manipulation on IGF-1 and IGF-1R

To the best of our knowledge, Western blot analysis is not common practice in evaluation of extracts of EGP. To validate our results of an increase in SIRT1 and IGFBP7 in the RES group, we tested the level of IGF-1R, which we previously found to be reduced under the same conditions by immunohistochemistry method (data not shown). Since immunohistochemistry is not reliable for quantitative analysis, we used this modified protocol for Western blots to follow quantitative changes in IGF-1R. As shown in Fig. 6 (B,C), there was a significant (50%) reduction in the IGF-1R protein in the RES group compared to the AL group. One day of refeeding was enough to increase the IGF-1R level back to normal (no significant difference between the CU and AL groups). Interestingly, the mRNA (Fig. 6A) showed the reverse effect to the protein, suggesting the possible involvement of miRNAs, which are increased by calorie restriction. The microarray platform we used did not identify any miRNA that could explain this observation.

In addition to the reduction in the IGF-1R levels in the EGP, the level of serum IGF-1 was also significantly reduced by calorie restriction (by ~80%; P<.05). One day of refeeding was enough to increase the level of IGF-1 back to normal (CU vs. AL; P=.1) (Fig. 6D).

4. Discussion

To the best of our knowledge, this is the first study showing that miRNAs are expressed in the EGP of mature animals and the first showing that miRNAs may respond to nutritional cues. Thus, miRNAs constitute a part of the complex sensing system designed to ensure that cells do not undergo periods of growth unless adequate levels of nutrients are available to produce the energy necessary to support that growth. Furthermore, this study demonstrates that there is a direct link between miR-140-3p, a chondrocytes-specific miRNA, and SIRT1 in the EGP. While calorie restriction reduces the level of miR-140-3p, it relieves the inhibition on the translation of SIRT1, and the level of SIRT1 protein is increased. SIRT1 was previously observed to be increased in a tissue-specific manner in response to long-term calorie restriction [27,29,30], but not in the context of linear growth (although SIRT1 knockout rodents are small [31]). SIRT1 was shown to have targets both in the nucleus and in the cytoplasm [32]; accordingly, immunohistochemistry showed that, in the EGP, SIRT1 can be identified in both regions.

The central role of miRNAs in mammalian development was first reported by Bernstein et al. [33] who found that development was arrested at E7.5 in Dicer-null mouse embryos. In addition, mice lacking Dicer in their cartilage had many skeletal defects during

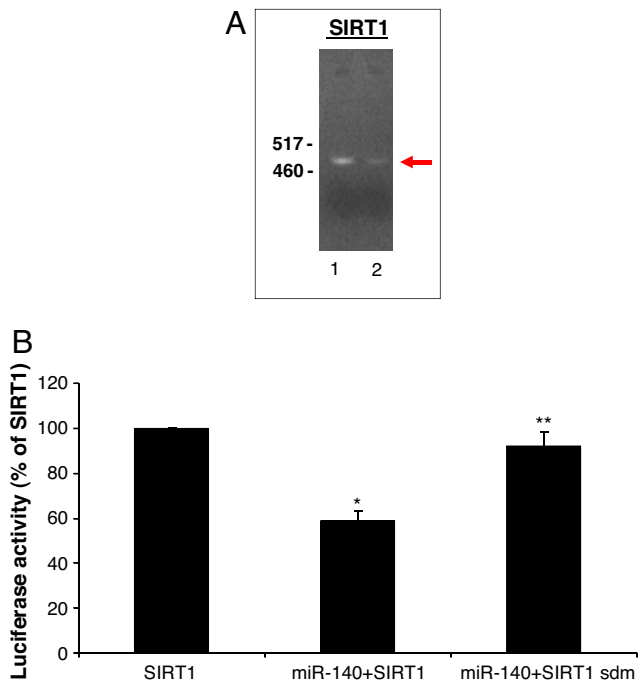

Fig. 5. Direct effect of miRNA-140 on the 3′UTR of SIRT1. (A) RT-PCR showing expression of the 3′UTR of SIRT1 in RNA extracted from the EGP: lane 1, genomic DNA; lane 2, cDNA from the EGP. (B) Dual luciferase reporter assay performed in HEK293 cells co-transfected with psiCHECK-SIRT1 and miR-Vec-140. The Renilla luciferase activity was normalized to that of the firefly and compared to cells transfected with the psiCHECK-SIRT1 alone. For a negative control, we used psiCHECK-SIRT1-sdm, which was not recognized by miR-140 (*P<.05 compared with SIRT1; **P<.05 compared with miR-140+SIRT1).

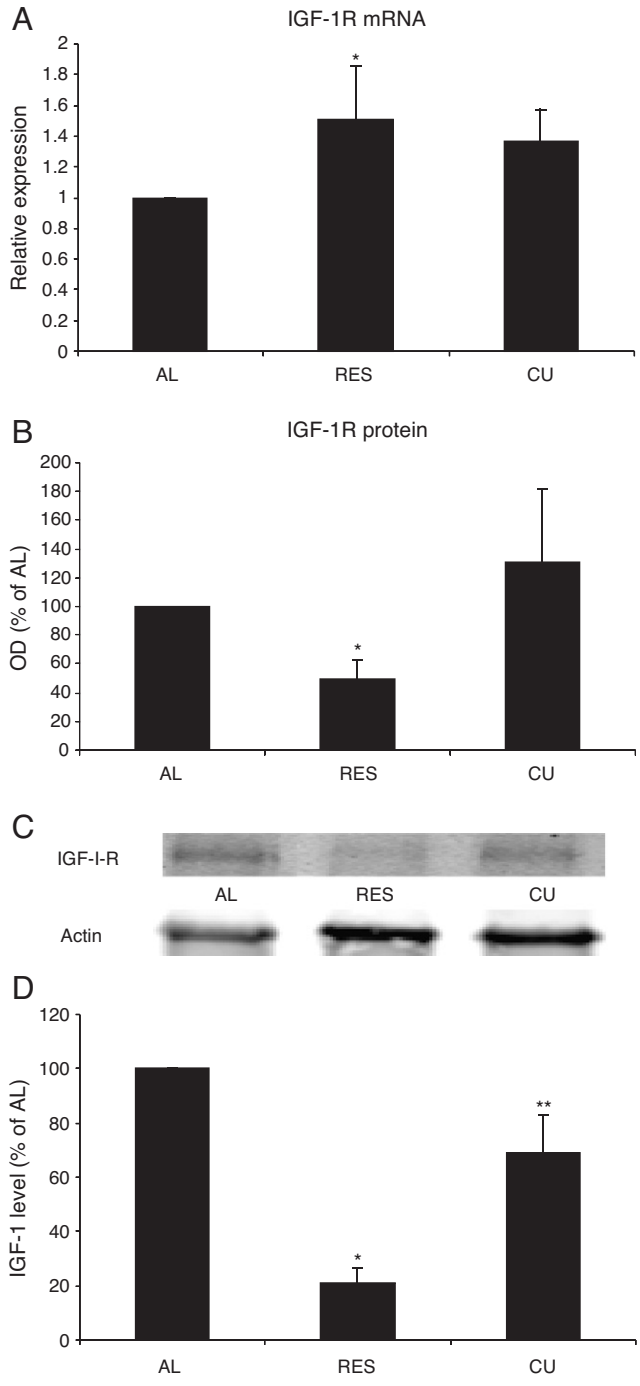

Fig. 6. Effect of food restriction on IGF-1R. (A) qPCR analysis of IGF-1R mRNA. (B) Densitometric analysis of the Western immunoblots for IGF-1R protein (presented in C). (C) Western immunoblots of IGF-1R with β-actin as reference. Data are given as mean±S.E. *P<.05 compared with AL. Results given are the average of three separate experiments. (D) Serum derived from rats of the three groups was analyzed for the presence of IGF-1 using radioimmunoassay. Data are given as mean±S.D. *P<.05 compared with AL; **P<.05 compared with RES.

development [34]. Dicer has been found to significantly affect limb size and morphogenesis, and its absence leads to a delay in the expression of limb-patterning genes [35]. In our study, a high-throughput miRNA microarray technology was used to identify the battery of miRNAs that are expressed in the mature EGP. Interestingly, of the miRNAs that Kobayashi et al. [34] found to be preferentially expressed in mouse chondrocytes, in the present study, only miR-196a was relatively highly expressed, whereas miR-202 and miR-496 were not. This discrepancy might be explained by species and age differences or by our selection of miRNAs on the basis of their level of expression and not their chondrocyte specificity.

The present study is also the first to demonstrate the effect of nutritional manipulation on miRNA expression. Interestingly, only few miRNAs were reduced by food restriction, and none was significantly increased. None of these miRNAs, except miR-140-3p, was previously reported to be associated with chondrocyte function [22,25,36–39]. Interestingly, although these miRNAs were identified in both the proliferative and hypertrophic zones, the effect of nutritional manipulation on miRNAs levels was evident mostly in the proliferative zone. We have previously shown that the proliferative zone is severely affected by nutritional restriction; there was a reduction in both cell numbers, zone height and BrdU-positive cells [16]. These results may suggest that the miRNAs are associated with cell proliferation. Indeed, miR-21 and miR-126 were previously reported to be involved in growth regulation [40,41].

The number of experimentally validated mRNA targets is still low, and the available computational programs predict somewhat different target lists, apparently because these algorithms take some similar and some unique characteristics of the miRNA-target interactions into account [25]. In addition, there is a discrepancy between the predicted and the *in vitro* validated targets (e.g., miR-126 and IGFBP7). As the expression of miRNA is tissue and time specific [36], effective regulation of transcript translation requires that miRNAs and their targets reside in the same microenvironment. In contrast to Nicolas et al. [25] who examined the effect of miRNA regulation on the mRNA level, we decided to follow the effect on protein levels [42]. Because of its extremely high level of expression, we decided to focus our attention on miR-140-3p, and we found that while the mRNA levels of SIRT1 were only marginally affected, a significant change in the protein levels was observed.

Calorie restriction induces a global change in gene expression [21]. Therefore, we sought to study the effect of calorie restriction on the epigenetics-associated targets of miR-140-3p: HDAC4 and SIRT1. HDAC4, a class II HDAC, was previously shown to regulate hypertrophy and skeletogenesis [37] by inhibiting the action of RUNX2, itself a transcription factor required for hypertrophy and osteoblast differentiation [43]. The calorie-restriction-induced reduction in the protein level of HDAC4 suggested that HDAC4 is not involved in nutrition-related growth attenuation. Therefore, it was not further examined in our study.

SIRT1 is a member of the sirtuin family of class III HDACs, highly conserved enzymes that utilize nicotinamide adenine dinucleotide (NAD+) to deacetylate a number of histone and nonhistone substrates. The founding member of this family, silent information regulator 2, promotes longevity in yeast by repressing gene expression and stabilizing chromatin. SIRT1 is involved in various nuclear events, such as transcription, DNA replication and DNA repair. It was found to regulate the proliferation, senescence and apoptosis of cells via the regulation of several transcription factors that govern metabolism and endocrine signaling, including PPAR-γ [44], PGC-1 α [45], FOXOs [46,47], Ku70, NF-kB, p300 and p53 [48,49].

SIRT1 is widely implicated in the response to calorie restriction in numerous tissues in a tissue-specific manner [27,29–30]. Calorie restriction and fasting increase the NAD+ level, which positively regulates the enzymatic activity of SIRT1. Some studies found that SIRT1 was induced by nutrient deprivation *in vitro* and after long-term calorie restriction *in vivo* in the brain, adipose tissue, kidney and liver [27]; others reported that levels increased in adipose tissue and muscle, but decreased in the liver [30]. We are the first, to the best of our knowledge, to show the presence of SIRT1 in the EGP. The increase in SIRT1 may explain the global shutdown observed under calorie-restricted conditions; this hypothesis, however, needs further verification.

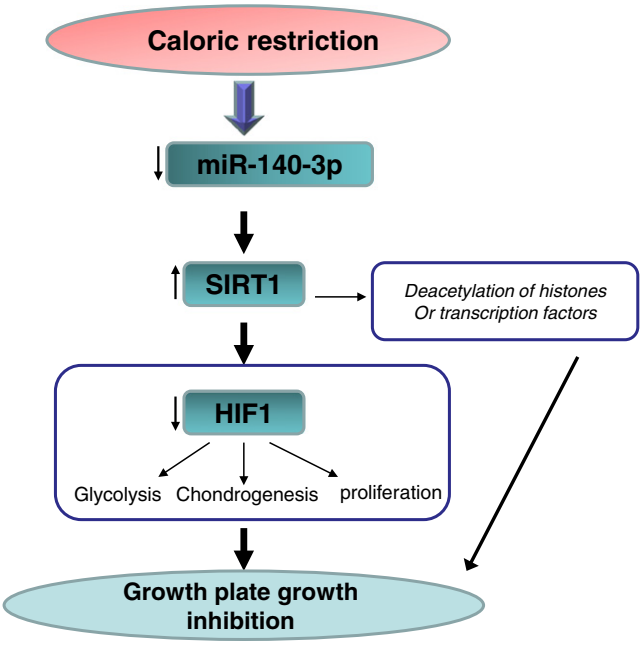

Fig. 7. Calorie restriction effect on the EGP — a suggested model. Calorie restriction reduces by an as yet unknown mechanism the level of miRNA-140-3p. This reduction relieves the inhibition on SIRT1 translation, leading to an increase in SIRT1 protein levels and a possible concomitant increase in deacetylation of HIF1α. The reduction in its level and activity leads to a reduction in glycolysis, chondrogenesis and proliferation. Deacetylation of additional proteins such as histones and transcription factors may contribute to the EGP growth attenuation.

In our previous publication, we reported that calorie restriction reduces HIF1α expression, protein level and activity [21]. In the present study, we found that SIRT1 is increased under the same conditions. The association between HIF1α and SIRT1 is supported by a recent publication of Lim and colleagues [50] showing that SIRT1 deacetylates HIF1α and reduces its activity. Another important factor in the response to nutritional modification is the mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine protein kinase and key regulator of cell metabolism which is inactivated when nutrient levels are inadequate. Its inhibition leads to protein synthesis inhibition, cell growth arrest, inhibition of HIF1α and glycolysis and autophagic protein degradation [51]. We therefore *suggest* the following model (Fig. 7): Calorie restriction reduces miR-140 levels by a still-unknown mechanism (involving mTOR?). This reduction relieves the translational inhibition of SIRT1 and leads to an increase in SIRT1 levels and a *possible* increase in its activity and, in turn, deacetylation and reduced activity of HIF1α and its downstream targets [21]. This model suggests a cross talk between nutrition, miR-140-3p, SIRT1 and HIF1α. It would be interesting to study the role of mTOR in this paradigm.

IGFBP7 (also termed mac 25/angiomodulin /IGFBP-related protein 1) is a secreted 27-kDa protein that binds IGF with a relatively weak affinity [52]. It plays a multifunctional role in regulating cellular proliferation, adhesion and angiogenesis and was shown to be involved in senescence and apoptosis. Findings of IGFBP7 down-regulation in several tumor cell lines and its high expression in senescent cells suggested that it may also exert tumor-suppressive or antiproliferative activity in normal tissue [52]. The recent finding of Lui et al. [53] of an increase in IGFBP7 level in the EGP with aging supports our finding of an increase in IGFBP7 in association with growth attenuation. What is intriguing is that only 1 day of refeeding was enough to reduce the level of IGFBP7 back to normal, implying that, during CU growth, the EGP returns to a "younger" stage of development [53]. However, the mechanism that explains the discrepancy between the effect on mRNA and protein level was not found in this study.

Negative energy balance leads to reduced plasma levels of GH, IGF-1, thyroid hormone, insulin and glucose and increases the levels of glucocorticoids. All these factors have direct effect on linear growth, and thus, the consequence of nutritional restriction is reduction in growth and body size. Interestingly, unlike well-fed children, malnourished children do not respond well to growth hormone (GH) treatment [54]. The lack of positive staining with the anti-GHR and anti-IGF-1R antibodies in the RES mice [16] and the marked reduction in IGF-1R in the EGP of RES rats by Western immunoblots in the present study, indicate that the EGP is rendered GH and IGF-1 resistant during food restriction. The remarkable reduction in IGF-1R protein level in the presence of an increase in its mRNA level is also suggestive of miRNA regulation; however, the panel we used failed to identify a miRNA that can explain this observation. Further investigation is needed to determine if this effect occurs as a consequence of changes in an as yet unidentified miRNA or another regulatory system. In contrast to the significant decrease in IGF-1R protein levels, food restriction induced a significant increase in IGF-1R mRNA levels, probably as a result of the marked reduction in serum IGF-1 levels [16,55].

To summarize, we have identified a set of miRNAs that are highly expressed in the mature mammalian EGP. Our results suggest that epigenetic mechanisms of miRNAs and HDACs from the SIRT family respond to nutritional cues. These findings may offer a possible explanation for some of the effects of food restriction on EGP growth. The mechanisms by which SIRT1 and miRNAs sense and respond to the change in nutritional status, and the role of SIRT1 in growth regulation will be further studied *in vitro*. Deciphering the role of epigenetic regulation in growth may open a new era of research and pave the way for the development of new treatments for children with growth disorders.

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References

[1] Holness MJ, Caton PW, Sugden MC. Acute and long-term nutrient-led modifications of gene expression: potential role of SIRT1 as a central co-ordinator of short and longer-term programming of tissue function. Nutrition 2010;26:491–501.
[2] He Z, Sontheimer EJ. "siRNAs and miRNAs": a meeting report on RNA silencing. RNA 2004;10:1165–73.
[3] Chen K, Rajewsky N. The evolution of gene regulation by transcription factors and microRNAs. Nature reviews 2007;8:93–103.
[4] Cuellar TL, McManus MT. MicroRNAs and endocrine biology. J Endocrinol 2005;187:327–32.
[5] Schickel R, Boyerinas B, Park SM, Peter ME. MicroRNAs: key players in the immune system, differentiation, tumorigenesis and cell death. Oncogene 2008;27:5959–74.
[6] Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. Nat Immunol 2008;9:839–45.
[7] Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, et al. A microRNA feedback circuit in midbrain dopamine neurons. Science 2007;317:1220–4.
[8] Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, et al. MicroRNA-143 regulates adipocyte differentiation. J Biol Chem 2004;279:52361–5.

[9] Zhang J, Jima DD, Jacobs C, Fischer R, Gottwein E, Huang G, et al. Patterns of microRNA expression characterize stages of human B-cell differentiation. Blood 2009;113:4586–94.
[10] Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, et al. A pancreatic islet-specific microRNA regulates insulin secretion. Nature 2004;432:226–30.
[11] Papagiannakopoulos T, Kosik KS. MicroRNAs: regulators of oncogenesis and stemness. BMC Med 2008;6:15.
[12] Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–66.
[13] Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. J Autoimmun 2009;32:189–94.
[14] Boersma B, Wit JM. Catch-up growth. Endocr Rev 1997;18:646–61.
[15] Gafni RI, Baron J. Catch-up growth: possible mechanisms. Pediatr Nephrol 2000;14:616–9.
[16] Gat-Yablonski G, Shtaif B, Abraham E, Phillip M. Nutrition-induced catch-up growth at the growth plate. J Pediatr Endocrinol Metab 2008;21:879–93.
[17] Maor G, Rochwerger M, Segev Y, Phillip M. Leptin acts as a growth factor on the chondrocytes of skeletal growth centers. J Bone Miner Res 2002;17: 1034–43.
[18] Gat-Yablonski G, Ben-Ari T, Shtaif B, Potievsky O, Moran O, Eshet R, et al. Leptin reverses the inhibitory effect of caloric restriction on longitudinal growth. Endocrinology 2004;145:343–50.
[19] Ben-Eliezer M, Phillip M, Gat-Yablonski G. Leptin regulates chondrogenic differentiation in ATDC5 cell-line through JAK/STAT and MAPK pathways. Endocrine 2007;32:235–44.
[20] Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS. Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. Genes Dev 2001;15:2865–76.
[21] Even-Zohar N, Jacob J, Amariglio N, Rechavi G, Potievsky O, Phillip M, et al. Nutrition-induced catch-up growth increases hypoxia inducible factor 1alpha RNA levels in the growth plate. Bone 2008;42:505–15.
[22] Tuddenham L, Wheeler G, Ntounia-Fousara S, Waters J, Hajihosseini MK, Clark I, et al. The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. FEBS letters 2006;580:4214–7.
[23] Rosenfeld N, Aharonov R, Meiri E, Rosenwald S, Spector Y, Zepeniuk M, et al. MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol 2008;26: 462–9.
[24] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods (San Diego, Calif 2001;25:402–8.
[25] Nicolas FE, Pais H, Schwach F, Lindow M, Kauppinen S, Moulton V, et al. Experimental identification of microRNA-140 targets by silencing and over-expressing miR-140. RNA 2008;14:2513–20.
[26] Vizioli MG, Sensi M, Miranda C, Cleris L, Formelli F, Anania MC, et al. IGFBP7: an oncosuppressor gene in thyroid carcinogenesis. Oncogene 2010;29: 3835–44.
[27] Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 2004;305:390–2.
[28] Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, et al. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 2006;124:1169–81.
[29] Kanfi Y, Peshti V, Gozlan YM, Rathaus M, Gil R, Cohen HY. Regulation of SIRT1 protein levels by nutrient availability. FEBS letters 2008;582:2417–23.
[30] Chen D, Bruno J, Easlon E, Lin SJ, Cheng HL, Alt FW, et al. Tissue-specific regulation of SIRT1 by calorie restriction. Genes Dev 2008;22:1753–7.
[31] McBurney MW, Yang X, Jardine K, Hixon M, Boekelheide K, Webb JR, et al. The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. Mol Cell Biol 2003;23:38–54.
[32] Jin Q, Yan T, Ge X, Sun C, Shi X, Zhai Q. Cytoplasm-localized SIRT1 enhances apoptosis. J Cell Physiol 2007;213:88–97.
[33] Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, et al. Dicer is essential for mouse development. Nat Genet 2003;35:215–7.
[34] Kobayashi T, Lu J, Cobb BS, Rodda SJ, McMahon AP, Schipani E, et al. Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. Proc Natl Acad Sci U S A 2008;105:1949–54.
[35] Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. Proc Natl Acad Sci U S A 2005;102:10898–903.
[36] Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, et al. MicroRNA expression in zebrafish embryonic development. Science 2005;309:310–1.
[37] Vega RB, Matsuda K, Oh J, Barbosa AC, Yang X, Meadows E, et al. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell 2004;119:555–66.
[38] Colnot C, Romero DM, Huang S, Helms JA. Mechanisms of action of demineralized bone matrix in the repair of cortical bone defects. Clin Orthop Relat Res 2005: 69–78.
[39] Eberhart JK, He X, Swartz ME, Yan YL, Song H, Boling TC, et al. MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. Nat Genet 2008;40:290–8.
[40] Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, et al. Endogenous human microRNAs that suppress breast cancer metastasis. Nature 2008;451: 147–52.
[41] Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 2005;65:6029–33.
[42] Pais H, Nicolas FE, Soond SM, Swingler TE, Clark IM, Chantry A, et al. Analyzing mRNA expression identifies Smad3 as a microRNA-140 target regulated only at protein level. RNA 2010;16:489–94.
[43] Colnot C. Cellular and molecular interactions regulating skeletogenesis. J Cell Biochem 2005;95:688–97.
[44] Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature 2004;429:771–6.
[45] Rodgers JT, Lerin C, Gerhart-Hines Z, Puigserver P. Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. FEBS letters 2008;582:46–53.
[46] Nemoto S, Fergusson MM, Finkel T. Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. Science 2004;306:2105–8.
[47] Jing E, Gesta S, Kahn CR. SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. Cell Metab 2007;6:105–14.
[48] Blander G, Guarente L. The Sir2 family of protein deacetylases. Annu Rev Biochem 2004;73:417–35.
[49] Cheng HL, Mostoslavsky R, Saito S, Manis JP, Gu Y, Patel P, et al. Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proc Natl Acad Sci U S A 2003;100:10794–9.
[50] Lim JH, Lee YM, Chun YS, Chen J, Kim JE, Park JW. Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. Mol Cell 2010;38:864–78.
[51] Tokunaga C, Yoshino K, Yonezawa K. mTOR integrates amino acid- and energy-sensing pathways. Biochem Biophys Res Commun 2004;313:443–6.
[52] Yamanaka Y, Wilson EM, Rosenfeld RG, Oh Y. Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. J Biol Chem 1997;272: 30729–34.
[53] Lui JC, Andrade AC, Forcinito P, Hegde A, Chen W, Baron J, et al. Spatial and temporal regulation of gene expression in the mammalian growth plate. Bone 2010;46:1380–90.
[54] Maes M, Maiter D, Thissen JP, Underwood LE, Ketelslegers JM. Contributions of growth hormone receptor and postreceptor defects to growth hormone resistance in malnutrition. Trends Endocrinol Metab 1991;2:92–7.
[55] Hernandez-Sanchez C, Werner H, Roberts Jr CT, Woo EJ, Hum DW, Rosenthal SM, et al. Differential regulation of insulin-like growth factor-I (IGF-I) receptor gene expression by IGF-I and basic fibroblastic growth factor. J Biol Chem 1997;272: 4663–70.